DEVELOPMENT AND DETECTION OF DOMINANCE AMONG LACTIC ACID SPOILAGE BACTERIA FROM CURED MEATS

By

Guopeng Zhang

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree of

MASTER OF SCIENCE

Department of Food Science
University of Manitoba
Winnipeg, Manitoba

National Library
of Canada

Acquisitions and
Bibliographic Services

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale
du Canada

Acquisitions et
services bibliographiques

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

0-612-32978-X

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

*****

COPYRIGHT PERMISSION PAGE

DEVELOPMENT AND DETECTION OF DOMINANCE AMONG LACTIC ACID SPOILAGE BACTERIA FROM CURED MEATS

BY

GUOPENG ZHANG

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree

of

MASTER OF SCIENCE

I hereby declare that I am the sole author of this thesis.

I authorize the University of Manitoba to lend this thesis to other institutions or individuals for the purpose of scholarly research.

Guopeng Zhang

Guopeng Zhang

I further authorize the University of Manitoba to reproduce this thesis by photocopying or by other means, in total or in part, at the request of other institutions or individuals for the purpose of scholarly research.

Guopeng Zhang

Guopeng Zhang

The University of Manitoba requires the signatures of all persons using or photocopying this thesis. Please sign below, and give address and date.

This thesis is dedicated to my mom for her unending love and support, which has given me the confidence and determination to pursue my goals.

Abstract

Spoiled vacuum-packaged sliced ham and corned beef samples were analyzed and both homofermentative lactobacilli and leuconostocs were found to dominate in the spoilage process (10^7-10^9 CFU/cm^2). The number of lactobacilli was consistently a little bit higher than the leuconostocs, but no more than one order. In corned beef packages, the bacterial number in the slime (purge) was one or two orders higher than on the meat surface. The organisms that formed ropy slime on the packaged meat lost their ropy slime-producing ability on agar plates. Sucrose addition to the formulation of corned beef did not change the dominance pattern of the LAB which was not surprising since all the strains recovered from the sucrose-added and control meat samples were able to ferment sucrose.

These isolates were identified as *Lactobacillus curvatus*, *Lactobacillus sake* and *Leuconostoc mesenteroides* ssp. *mesenteroides* based on their morphology, key biochemical reactions and the API 50 CHL test results. Pulsed-field gel electrophoresis of the genomic DNA with *Sma*I digestion distinguished all the LAB strains under study. Nonetheless, it was found that the PFGE (*Sma*I digestion) technique by itself was not a suitable method to identify species of *L. curvatus* or *L. sake*. A phylogenetic analysis of 7 *Leuconostoc* strains using data generated by PFGE revealed that two bacteriocin-producing strains (both isolated from commercial meats from Quebec) had a close genetic relationship. A "composite-simultaneous" restriction endonuclease digestion method was developed which enabled differential enumeration of LAB strains in mixtures by PFGE.

Among 20 LAB strains isolated from commercial meats, two *Lc. mesenteroides*

strains and one *L. sake* strain were able to produce bacteriocin and two *L. sake* strains (No.6 and No.9) plus one *L. curvatus* strain (No.10) were found to be sensitive to these bacteriocins. These bacteriocin-producing bacteria produced bacteriocin when growing on agar surfaces but not when growing in broth.

$NaNO_2$ (<200 ppm) had little effect on the growth of spoilage LAB strains in MMRS broth at 6°C. NaCl (>4%) had a significant inhibitory effect on the growth capability of *Lc. mesenteroides* and *L. sake* strains at 6°C but had no effect on *L. curvatus* strains. Lower initial pH of the MMRS broth also had a significant inhibitory effect on *Lc. mesenteroides* and *L. sake* but had little effect on *L. curvatus*. Lactobacilli generally grew better than *Lc. mesenteroides* at pH 5.5. Different incubation temperatures had a significant effect on the growth of all 3 species. It appeared that *L. curvatus* and *L. sake* grew faster at 12°C than *Lc. mesenteroides*. It was also noted that there was a similar response for strains within the same species to the challenges of NaCl, $NaNO_2$, pH and temperature. When present in mixed cultures, *Leuconostoc* strains did not grow well at 2°C and at an initial pH of 5.5, compared with lactobacilli. At normal cured meat pH (6.0 and 6.5) and higher temperatures (6°C and 12°C), dominant bacteria always developed from the originally larger bacterial population. When leuconostocs and lactobacilli were present in equal initial numbers, lactobacilli dominated at 2°C and pH 5.5. In *Lc. mesenteroides* and *L. curvatus* mixtures, *Lc. mesenteroides* dominated at 6°C ($pH \geq 6.0$) while *L. curvatus* dominated at 12°C ($pH \geq 6.0$). In *Lc. mesenteroides* and *L. sake* mixtures, there was no significant difference in numbers between *Lc. mesenteroides* and *L. sake* at

temperatures≥6°C and pH≥6.0. No substantial inhibitory effect of bacteriocin was observed in the mixed cultures.

Acknowledgments

I would like to express my great appreciation to my advisor, Dr. R. A. Holley for all his expertise, guidance, patience and continuous support during my study. I would also like to thank Dr. G. Blank and Dr. G. R. Klassen for their participation on my examining committee.

I'm also deeply grateful to Dr. S. D. Arntfield, Dr. M. A. H. Ismond, Dr. G. Crow, Mr. J. W. Rogers, Dr. B. Fristensky, Dr. P. Loewen and Mr. R. Roller for providing me expertise in their respective areas. Financial support from the UM Graduate Fellowship and D. L. Campbell Graduate Fellowship were greatly appreciated.

Thank-you's are also extended to all my friends, who supported me over the past two years. To the UM boys and girls. Your friendship and support made my life in Canada colorful and unforgettable.

Last, but certainly not least, I would like to thank Yinghua for her encouragement and assistance in completing my thesis.

Table of Contents

Chapter 3

Chapter 4

Chapter 5

List of Tables

List of Figures

List of Abbreviations

APT	All purpose Tryptone (Tween) medium developed by J.B. Evans and C. F. Niven (1951)
ATCC	American Type Culture Collection
CFU	colony forming unit
ES	a buffer containing EDTA and sarcosine only
ESP	a buffer containing EDTA, sarcosine and proteinase K.
HGMF	hydrophobic grid membrane filtration
HHD	homofermentative-heterofermentative differential medium developed by McDonald et al. (1987)
LAB	lactic acid bacteria
LMP	low melting point
MES	morpholinoethane sulphonic acid
MMRS	modified MRS medium
MRS	medium devised by J. C. de Man, M. Rogosa and M. E. Sharpe (1960) for the growth and maintenance of lactic acid bacteria
MRSD	MRS differential medium developed by R. A. Holley and G. E. Millard (1988)
PFGE	pulsed-field gel electrophoresis
PMSF	phenylmethylsulfonyl fluoride
PROC GLM	general linear models procedure
RAPD	randomly amplified polymorphic DNA

RESTML	restriction sites maximum likelihood method
SDS-PAGE	sodium dodecyl benzene sulfonate-polyacrylamide gel electrophoresis
TBE	a buffer containing TrisHCl, borate and EDTA
TE	a buffer containing TrisHCl and EDTA
USDA	United States Department of Agriculture

List of Appendices

CHAPTER 1

INTRODUCTION

Vacuum-packaged cooked cured meat products such as ham and bologna comprise a major portion of processed meats for sale at retail and have become very popular over the years because of their long shelf life (usually 2-7 weeks) and their ready-to-eat convenience. Lactic acid bacteria (LAB) are a major component of the microbial population found on various types of vacuum-packaged cured meats (Mol et al., 1971, Borch et al., 1997). Homofermentative lactobacilli and leuconostocs are two major types of LAB repeatedly responsible for premature spoilage of vacuum-packaged cooked cured meat products (Holley, 1997; Korkeala and Bjorkroth, 1997), which leads to considerable financial loss to manufacturers.

Lactobacillus curvatus (*L. curvatus*), *Lactobacillus sake* (*L. sake*) and *Leuconostoc mesenteroides* ssp. *mesenteroides* (*Lc. mesenteroides*) are three LAB species frequently reported to dominate in the spoilage of vacuum-packaged cured meats (Dykes et al., 1994). Since this group of bacteria (especially *L. curvatus* and *L. sake*) share similar morphological and biochemical characteristics, strain identification is problematic. Among several methods used to identify LAB strains, pulsed field gel electrophoresis (PFGE) of restriction endonuclease-digested genomic DNA has proven to be reliable for strain characterization due to its high resolution and excellent reproducibility (Bjorkroth et al., 1996).

No information is available which predictably explains reasons for dominance of

Lactobacillus or Leuconostoc strains during spoilage of vacuum-packaged cooked cured meat products. Such understanding why lactobacilli or leuconostocs dominate in the spoilage process would be very helpful for prediction of spoilage and manipulation of the dominance patterns in order to extend the shelf life of meat products. Factors such as pH, temperature, NaCl and $NaNO_2$ concentration, bacteriocin production and resistance may contribute to the dominance of these two genera.

The objective of this research was two fold. First, to isolate and characterize LAB strains from spoiled meat samples using conventional techniques. PFGE was also applied and evaluated for its usefulness in strain identification. Second, to conduct individual inhibitory factor tests and mixed-culture dominance tests (*in vitro*) to evaluate pH, temperature, NaCl and $NaNO_2$ concentrations, bacteriocin production and resistance as factors influencing the dominance of these spoilage LAB. In addition, we wished to test the hypothesis that lactobacilli and leuconostocs compete equivalently when present in equal number during challenges with these factors and that dominant bacteria develop from the larger initial bacterial population.

CHAPTER 2

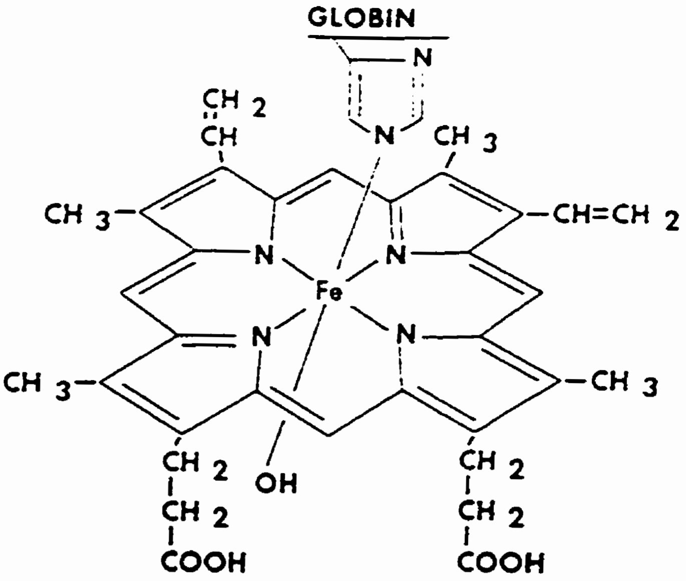
LITERATURE REVIEW

2.1. Microbiological Spoilage of Vacuum-Packaged Cooked Cured Meats

2.1.1. Cooked Cured Meat Products

Meat contains a wide variety of essential nutrients. Because of its nutritiousness and deliciousness, meat makes up a major part of the human diet in almost all nations. Meat products can be divided into fresh meats and processed meats: fresh meats include fresh cuts and other meat separated from animal carcasses without further thermal or chemical treatments. Processed meats include sausages, non-comminuted meats and restructured meats. Sausages can be made from coarsely ground meat or meat emulsions, while non-comminuted meats include those whole cuts such as bacon and ham. Restructured meats are meat pieces held together by proteinaceous extracts from meat.

Because meat has a very high water content (~70%), it is an extremely perishable product compared to some plant products such as grains. Preservation of meat by drying has been practiced for thousands of years. Besides drying directly by removal of water, the addition of salt was often used. The latter was known originally as "curing" (Townsend and Olson, 1987). In ancient time, by inadvertent use of impure sodium chloride containing nitrate or nitrite, a pleasant pink meat color was achieved as the result of conversion of myoglobin to nitrosomyoglobin (Figure 1). Nowadays, "cure" directly refers to sodium nitrite or potassium nitrite used in meat curing and the term "meat curing" eventually came



Myoglobin

$$\text{Nitrate} \xrightarrow[\text{organisms}]{\text{nitrate reducing}} \text{Nitrite} \tag{1}$$

$$\text{Nitrite} \xrightarrow[\text{absence of light and air}]{\text{favorable conditions}} \underset{\text{nitric oxide}}{\text{NO}} + \underset{\text{water}}{H_2O} \tag{2}$$

$$\underset{\text{nitric oxide}}{\text{NO}} + \underset{\text{myoglobin}}{\text{Mb}} \xrightarrow[\text{conditions}]{\text{favorable}} \underset{\substack{\text{nitric oxide}\\ \text{metmyoglobin}}}{\text{NOMMb}} \tag{3}$$

$$\text{NOMMb} \xrightarrow[\text{conditions}]{\text{favorable}} \underset{\text{nitric oxide myoglobin}}{\text{NOMb}} \tag{4}$$

$$\text{NOMb} + \text{Heat} + \text{Smoke} \rightarrow \underset{\substack{\text{Nitroso-hemochromogen}\\ \text{Stable pink pigment}}}{\text{NO—hemochromogen}} \tag{5}$$

Figure 1. Structure of myoglobin and the mechanism of color change in meat curing. (Pearson and Tauber, 1984)

to be understood as the addition of salt, sugar, nitrate or nitrite, as well as various spices, ascorbates and phosphates (Townsend and Olson, 1987).

The function of nitrite in meat curing is not limited to stabilizing the pink color of lean meat. It also contributes to the characteristic flavor of cured meats, inhibition of a number of food poisoning and spoilage microorganisms, and in another aspect, in terms of retarding the development of rancidity (Pearson and Tauber, 1984). Brooks et al.(1940) first reported that the characteristic bacon and ham flavors were due to the addition of nitrite. However, the search for the specific compounds developed which are responsible for cured meat flavor is still going on (Townsend and Olson, 1987). Sodium/potassium nitrite has a significant antibacterial affect on *Clostridium botulinum*, an anaerobic spore-forming microorganism that produces botulinum toxin, which is potent and lethal to humans. The bactericidal activity of nitrite increases with temperature (Roberts et al., 1981). Other research has shown that nitrite delays the development of oxidative rancidity (Watts, 1954). The mechanism involves two important features. Iron ions from heme compounds in meat act as a catalyst for lipid oxidation. The reaction associated with nitrite in forming cured meat pigments retains the iron in the heme, often in the reduced form (Fe^{2+}), so the iron is inactive as a catalyst for lipid oxidation.

However, improper usage of nitrite has generated adverse consumer opinion regarding cooked cured meats. Before 1945 there was little concern about the amount of nitrite or nitrate in relation to the human diet. At first, the acute toxicity of nitrate as well as the relationship of nitrate and methemoglobinemia were of concern (Hill, 1991). The hypothesis that nitrate exposure might be implicated in the development of human cancer stemmed from the knowledge that N-nitroso compounds form a family of highly carcinogenic compounds that can be generated in foods treated with nitrite or nitrate at high concentration and at elevated temperature. Yet N-nitroso compounds have not been found in cured meats prepared according to the current regulations (Hill, 1991). The US. Federal limitation of nitrite in finished products is 200 ppm, but only 120 ppm is allowed in bacon (USDA, 1975). In cooked cured products, the residual nitrite found is only about 50 ppm (Townsend and Olson, 1987).

Cured meat products may be manufactured from whole cuts (non-comminuted meat) such as corned beef, or be restructured from smaller meat pieces such as molded sliced ham. Sausages also can be one form of cured meat if nitrate/nitrite is added. These products include dry cured and smoked sausages, plus cooked, cured and smoked sausages. Bologna is such a type of sausage with an emulsion base. Canned products such as cooked ham and luncheon meat with vegetables or added pasta are other types of cured meats.

In the meat curing process, meats are first treated with NaCl to yield a final concentration of 2-3% (w/v). Sodium nitrite is added as the curing agent to a final concentration of less than 200 ppm (ug/g). Sugar (mainly glucose, sucrose or lactose) is another ingredient added to the meat and the concentration is around 1% (w/v). Texture improvers, binders, spices and smoke may be added. Phosphate or lactate may also be added as humectants to improve the water-holding ability and reduce water activity (a_w) at the same time. Ascorbates such as sodium erythorbate may be added to speed up the cure reaction and color development of meat products. Starch, flour and milk ingredients may be used as fillers (Townsend and Olson, 1987).

After curing (dry or pickle curing), the meats are filled in cans, molds, casings or thermo-resistant plastic pouches and cooked to an internal temperature of 65-75°C (Pearson and Tauber, 1984) to yield products which are generally stable under refrigerated conditions. Canned products are given a more severe heat treatment (>66°C internal) and are usually shelf-stable at room temperature if less than 3 lbs. After heat treatment and chilling, the cooked cured meat products may be sliced and repackaged (usually by vacuum-packaging) in the original processing plant or at the retail outlet.

Vacuum-packaging of cooked cured meat products has become very popular. By excluding air from the meat with O_2 impermeable (barrier) pouches, an anaerobic environment is generated. Advantages of vacuum-packaging include hygienic handling of products, increased shelf-life, good labeling properties and better decontamination possibilities by postprocessing treatments such as irradiation. There are also some disadvantages of vacuum-packaging: equipment and material-related costs, drip (purge or exudate)formation, poor separation of slices (if it is a sliced product) and packaging waste (Korkeala and Bjorkroth, 1997).

2.1.2. Microbiological Spoilage of Vacuum-Packaged Cured Meats

2.1.2.1. Microbiology of fresh meats

Many species of microorganisms are able to grow on fresh meat. Most of the bacteria found on skinned carcasses are Gram-positive mesophiles such as *Micrococcus*, *Staphylococcus* and *Bacillus* with the presence of a few Gram-negative mesophilic bacteria (Egan and Roberts, 1987).

As the carcasses are moved into the chill room at near 0°C, a selective environment is developed which favors the growth of psychrotrophic bacteria. Three genera usually predominate. They are *Pseudomonas*, *Moraxella* and *Acinetobacter*. These microorganisms are Gram-negative, strictly aerobic rods that can grow at low temperatures around 0°C (Gill, 1986). However, all of these bacteria are sensitive to heat and require a high a_w to grow. They are not normally associated with any human disease or food poisoning. Among these three genera the *Pseudomonas* strains grow most rapidly and will eventually dominate in the chilled fresh meats.

In addition to those Gram-negative rods mentioned above, other microorganisms may also grow on the moist surface of fresh meat. These include *Lactobacillus*, *Micrococcus*, *Aeromonas*, psychrotrophic *Enterobacteriaceae* as well as *Brochothix thermosphacta* (Sneath and Jones, 1976).

When the oxygen surrounding fresh meats is eliminated by vacuum-packaging or modified atmosphere (MA) packaging (100% CO_2) at low temperatures, the microflora will be dominated by LAB, mainly *Carnobacterium* spp., *Lactobacillus* spp. and *Leuconostoc* spp. (Shaw and Harding, 1984). *Carnobacterium* spp. dominate at -1.5°C on pork stored in 100% CO_2, while homofermentative *Lactobacillus* spp. dominate at 4°C and 7°C in these products (Borch et al., 1996).

2.1.2.2. Microbiology of vacuum-packaged cured meats

The spoilage flora of cured meats is very different from fresh meats. Salts and nitrite in the curing formula inhibit the growth of *Pseudomonas*, *Moraxella* and *Acinetobacter* but favor the growth of lactic acid bacteria (LAB), *Brochothrix thermosphacta* and some *Enterobacteriaceae* (Allen and Foster, 1960; Nielsen, 1983). Vacuum-packaging and the higher pH of cured meat slows down the growth of *Brochothrix thermosphacta* which is a facultative anaerobe. Proper storage temperatures (<6°C) will also eliminate the predominance of psychrotrophic *Enterobacteriaceae* because these microorganisms usually do not grow well at 6°C or below.

During the refrigerated storage of vacuum-packaged cooked cured meats, the growth of psychrotrophic LAB (mainly *Lactobacillus*, *Leuconostoc* and *Carnobacterium* spp.) is favored because they are salt tolerant and not very sensitive to nitrite (Castellani and Niven, 1955; Reuter, 1970; Mol et al., 1971). *Carnobacterium* spp. usually dominate at very low temperatures such as -1.5°C. However, the common storage temperature (4-9°C) favors the growth of other LAB such as lactobacilli. *Carnobacterium* spp. also can not survive the great amount of acid produced during meat storage, so this bacterium is not an important microbial component of vacuum-packaged cured meats (von Holy et al., 1991). A subsequent study from the same laboratory showed that dominant organisms in these type of meat products are either *Lactobacillus* or *Leuconostoc* spp. (Dykes et al., 1995). Species of *Lactobacillus curvatus* and *Lactobacillus sake* have often been shown to form the most important spoilage population of vacuum-packaged cooked meat products (Dykes and von Holy, 1994; Holzapfel and Gerber, 1986). In addition to these lactobacilli, *Leuconostoc mesenteroides* ssp. *mesenteroides* has also been frequently observed to form the main spoilage population in vacuum-packaged cured meat products (Dykes et al., 1994; Yang and Ray, 1994; Korkeala and Bjorkroth, 1997).

These spoilage organisms are not thermo-resistant (Franz and von Holy, 1996) and the initial number of spoilage LAB in the meat products is generally very low immediately after cooking. Makela and Korkeala (1987) found that the skins of cooked ring sausages were relatively free of lactobacilli after cooking, with only 2 out of 30 samples showing the presence of one organism per 10g of skin. The spoilage of cooked products mainly results from contamination after cooking (Makela and Korkeala, 1987) and microorganisms may gain entry during the slicing and vacuum-packaging operations. The contamination may come from air, processing equipment or from the hands of workers (Korkeala and Bjorkroth, 1997). It was found that the practice of "co-slicing", which is popular at both retail and processing plants may cause recontamination of cooked meats by transfer of spoilage LAB from dry fermented sausages to vacuum-packaged sliced meats (Holley, 1997).

2.1.2.3. Sensory and physical changes during spoilage

2.1.2.3.1. Sourness

Sour aroma and sour taste are the major offensive sensory qualities produced as a result of spoilage of vacuum-package cured meats (Korkeala et al., 1989; von Holy et al., 1991). Spoilage LAB produce lactic acid or acetic acid as end products of sugar metabolism. When the level of lactobacilli reached 5×10^7 CFU/g of the surface layer, lactic acid concentration increased sharply (Korkeala et al., 1990). The initial pH of cured meats ranges from 6.0 to 6.5 (Pearson and Tauber, 1984). Once the LAB reach a population of 5×10^7 CFU/g, the pH decreases sharply and meat samples become spoiled when the pH falls below 5.8 to 5.9 (Korkeala et al., 1990). Very low pH values such as 4.6 to 5.5 were also observed in spoiled vacuum-packaged cured meats (von Holy et al., 1991). Nonetheless, pH drop usually occurs after there are visible signs of bacterial spoilage (i.e. development of slime).

2.1.2.3.2. Gas formation

Blown-packages are a common form of spoilage with vacuum-packaged cured meat products. A major part of the gas is CO_2 which is a metabolic by-product of the heterofermentative LAB, such as *Leuconostoc* spp. (Ahvenainen et al., 1990). Excessive CO_2 produced in packages may lead to the leakage of the package and destroy the strict anaerobic environment surrounding the meat, thus altering the microbiological spoilage pattern. The CO_2 concentration in vacuum packages has been shown to be initially less than 10%, but increased sharply, to 40~60%, when lactobacilli exceeded 6.4×10^6 CFU/g (Korkeala et al., 1987). Yang and Ray (1994) observed that cured meats they studied were exclusively spoiled by *Leuconostoc* spp.. Gassing is probably the most important premature cause of spoilage of vacuum-packaged cured meats because it occurs at relatively low numbers of bacteria/cm^2.

2.1.2.3.3. Purge formation

Purge, exudate, slime or milky fluid is frequently observed to accumulate in packages of spoiled vacuum-packaged cooked cured meat products (Kempton and Bobier, 1970). Products are deemed as unacceptable when purge formation is very copious and covers the meat surface (Borch and Neibrink, 1989). The appearance of the purge changes from transparent to white or milky due to the pH drop (Ahvenainen et al., 1990).

2.1.2.3.4. Ropy slime formation

Ropy slime formation on vacuum-packaged cooked sausages was frequently observed in Finland during the 1980's (Korkeala et al., 1988). Ropy slime often forms before the product expiry date when the LAB are still in the logarithmic growth phase and before the pH of the product decreases (Korkeala and Bjorkroth, 1997). The presence of elastic long polysaccharide ropes can be observed when separating the sausages or slices. The appearance of the ropy slime is very undesirable and is clearly different from the slimy bacterial mass (Korkeala and Bjorkroth, 1997).

Based on DNA-DNA homology, 4 groups of *L. sake* strains and one *Leuconostoc gelidum* strain were identified as the cause of ropy slime formation (Makela et al., 1992) in a series of spoiled meats. One group of *L. sake* strains was found to be the most offensive ropy slime producer (Bjorkroth and Korkeala, 1996). Pulse-field gel electrophoresis (PFGE) of restriction endonuclease (*Sma*I and *Avr*II) digested genomic DNA analysis was conducted and this procedure successfully characterized 4 sub-groups of ropy slime-producing *L. sake* strains (Bjorkroth et al., 1996). Contamination of sausages by these ropy slime-producing LAB was found to occur after cooking, during chilling, handling, slicing and packing, because these organisms do not normally survive the internal sausage cook temperature of 68°C (Makela et al., 1992).

2.1.2.3.5. Asymmetric distribution of LAB during spoilage

Another interesting issue associated with the spoilage of vacuum-packaged cooked cured meat products is the asymmetric distribution and growth of spoilage LAB in packages, or "surface layer spoilage". Surface spoilage phenomenon not only occurs in vacuum-packaged whole meats but also in sliced products (Holley, 1997). Korkeala and Lindroth (1987) found that the predominant growth of bacteria at the meat surface was due to the greater a_w at the surface layer of the meat. However, this relationship was not established in the study by Holley et al. (1996). It was suggested the microbial distribution difference might result from the higher O_2 concentration at the meat surface inside the barrier package film (Bell and Gill, 1982; Holley et al., 1996). Slow diffusion of oxygen through the packaging film during storage may be the cause, especially when the barrier films are stretched during thermo-forming of the package (Holley, 1997). In the investigation with vacuum-packaged cooked ring sausages, 5×10^8 CFU/g LAB were found in the surface layer of the meat, but in the center of the sausage the bacterial load was only about 10^2-10^3 CFU/g, (Korkeala and Lindroth, 1987).

LAB, more specifically, homofermentative lactobacilli and leuconostocs form the major spoilage flora of vacuum-packaged cured meats. Sometimes this type of spoilage causes significant financial loss to the manufacturers. However, we should also be aware that the predominance of these LAB may initially serve a protective function by excluding other spoilage and pathogenic species (Holley, 1997).

2.2. Biochemical and Growth Characteristics of Spoilage LAB

L. curvatus, *L. sake* and *Lc. mesenteroides* make up the major spoilage flora of vacuum-packaged cooked cured meat products (Dykes et al., 1995). These microorganisms belong to a group of Gram-positive, catalase negative non-sporing micro-aerophilic bacteria whose main fermentation product from carbohydrates is lactate (Kandler, 1983). This group of bacteria, LAB, includes the genera *Carnobacterium*, *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus*. Lactic acid bacteria characteristically lack cytochromes and are dependent on substrate level phosphorylation during sugar fermentation for energy (Charteris et al., 1997). They are subdivided on the basis of their sugar utilization pathways and metabolic products (Kandler and Weiss, 1986) into homofermentative and heterofermentative LAB. Homofermentative LAB produce primarily lactic acid from hexose. Heterofermentative LAB can produce CO_2, lactic acid, acetic acid, ethanol, and mannitol from hexoses (McDonald et al., 1987).

The genus *Lactobacillus* contains a variety of homo- and hetero-fermentative species. However, the two most important spoilage lactobacilli, *L. curvatus* and *L. sake*, are both homofermentative (facultative heterofermentative) while *Leuconostoc* species are heterofermenters (Schillinger and Lucke, 1987, Stiles and Holzapfel, 1997).

2.2.1. *L. curvatus* and *L. sake*

Under the microscope, both *L. curvatus* and *L. sake* are usually nonmotile rods with rounded ends, generally 1-3 µm in length, occurring singly, in pairs or in short chains (Kandler and Weiss, 1986). *L. sake* cells are usually slightly thicker than *L. curvatus* cells (0.7-0.9 µm and 0.6-0.8 µm, respectively) and *L. curvatus* sometimes occur as curved rods but in general it is hard to tell this difference. Neither species is usually able to grow at 45°C but many strains tested are able to grow at 2-4°C, which enables their predominance in refrigerated vacuum-packaged cooked cured meat products (Kandler and Weiss, 1986). A DNA-DNA homology study revealed that *L. curvatus* and *L. sake* are closely related to each other. They share 40-50% homology. The mol% G+C of the DNA of these two species are both 42-44 (T_m). However, recent study of these two organisms showed that *L. sake* and *L. curvatus* are two well-defined species (Klein et al., 1996).

Data from Kandler and Weiss (1986) showed that 90% of *L. sake* strains investigated were amygdalin, arabinose, melibiose, sucrose and trehalose positive while 90% of *L. curvatus* strains showed negative results in these sugar fermentations. However, in an investigation by Schillinger and Lucke (1987), among these sugars only the melibiose fermentation profile of these two organisms was consistently reported (Kandler and Weiss, 1986; Hugas et al., 1993). From the results of Schillinger and Lucke, it was also found that 80% of *L. curvatus* strains could utilize maltose but 86% of *L. sake* did not. The arginine utilization test is another informative measure to differentiate *L. curvatus* from *L. sake*. Schillinger and Lucke (1987) found 88% of *L. sake* strains under study showed positive results but only 35% of *L. curvatus* strains could utilize arginine. Hugas et al. (1993) found that all the *L. sake* strains investigated were arginine positive while only 8% of *L. curvatus* strains possessed this property. In an investigation of spoiled retail delicatessen meats, all 6 *L. sake* strains isolated were found to be arginine positive and all 3 *L. curvatus* strains were negative (Holley et al., 1996). Arginine deamination broth is commonly made according to Niven et al. (1942) or Schillinger and Lucke (1987).

Addition of 0.03 or 0.05% glucose was suggested to yield more consistent results (Hitchener et al., 1982; Hugas et al., 1993).

2.2.2. *Lc. mesenteroides*

Lc. mesenteroides has spherical but often lenticular cells with a diameter of about 0.5 μm. The cells usually occur in pairs and chains and are nonmotile (Garvie, 1986). When cultured in broth, cells are elongated and can be mistaken for rods, appearing morphologically closer to the lactobacilli than to the streptococci (Garvie, 1986). That is the reason that morphologically, sometimes it is hard to differentiate leuconostocs from lactobacilli since they may both show coccobacillus shape. The chain length of leuconostocs varies with strains (up to 20 cells/chain). This may generate a problem when quantifying cell concentrations by plating on agar plates since the actual number can be one order higher than the colony forming units visualized on the plates. During an investigation of a *Lc. mesenteroides* strain in milk, the chains of cells were successfully disrupted mechanically using an Ultra-Turrax® T25 homogenizer for 30 seconds at 20,000 rpm before the cell suspensions were plated on agar medium (Bellengier et al., 1997).

Leuconostoc species are not able to hydrolyze arginine (Schillinger and Lucke, 1987) and thus can be differentiated from most heterofermentative lactobacilli. *Lc. mesenteroides* can produce a characteristic slime of dextran from glucose and its production is favored by growing at 20-25°C (Garvie, 1986). Unlike lactobacilli, this organism does not like very low temperatures. The growth temperatures of leuconostocs normally range from 10 to 37°C with the optimum at 20-30°C (Garvie, 1986).

2.2.3. Biochemical Identification of Spoilage LAB

A presumptive identification scheme was developed by Schillinger and Lucke (1987), (Figure 2). The Gram-positive, catalase negative, non-spore forming cocci or rods isolated from meats were first tested for CO_2 production from glucose (heterofermentivity). Gas (CO_2) production was observed in MRS broth (citrate omitted) containing inverted Durham vials. Positive strains were further tested in arginine hydrolysis broth. Arginine test-negative cocci were leuconostocs. Arginine test-negative rods or -positive organisms were heterofermentative lactobacilli. Rods not producing gas were homofermentative lactobacilli and cocci could be *Pediococcus*, *Lactococcus*, *Streptococcus* or *Enterococcus* spp. (Schillinger and Lucke, 1987).

Further separation of these *Lactobacillus* and *Leuconostoc* isolates into species can be done by using the API 50 CHL identification system (bioMerieux SA, Marcy-l'Etoile, France). The API 50 system provides 50 microtubes each containing an anaerobic zone (the tube portion) for sugar fermentation study and an aerobic zone (the cupule portion) for the study of oxidation and assimilation. Anaerobic conditions can be achieved by overlaying the cupule portion of the microtubes with sterile mineral oil. Forty nine tubes (plus one as control) contain defined amounts of different lyophilized carbohydrates or their derivatives. A sophisticated identification table with computerized database can be used for species determination (Instruction manual, API 50 CHL, bioMerieux SA, France).

2.2.4. Media for Differential Enumeration of Spoilage LAB

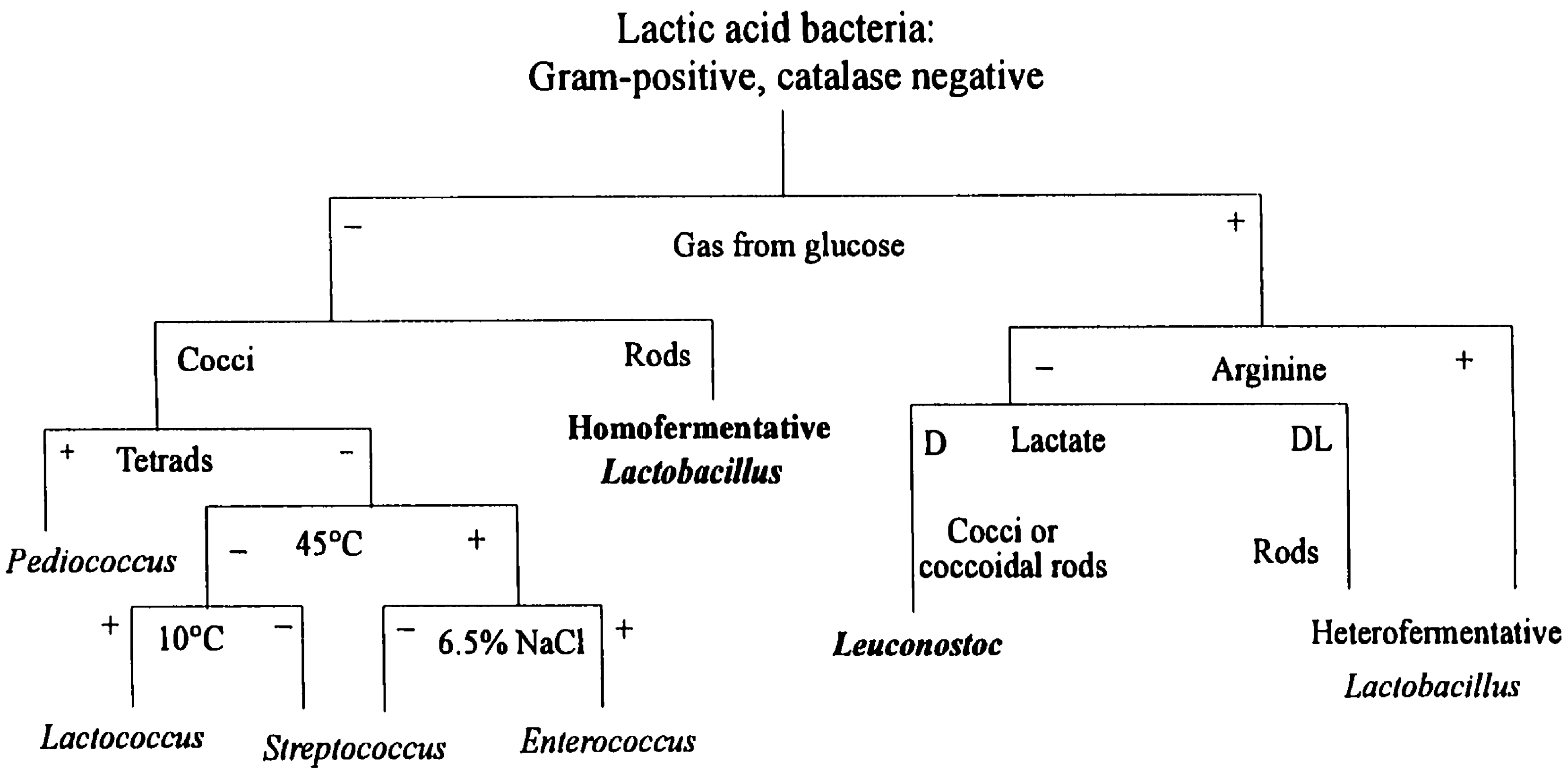


Figure 2. Differentiation scheme for lactic acid bacteria isolated from meats. 10°C: growth at 10°C; 45°C: growth at 45°C; 6.5% NaCl: growth in presence of 6.5% NaCl; arginine: hydrolysis of arginine; lactate: production of lactate; D: the D-lactate isomer recorded makes up 90% or more of the total lactic acid; DL: 25-75% of total lactic acid are of the L-configuration. (Schillinger and Lucke, 1987).

Although de Man-Rogosa-Sharpe (MRS) agar and All Purpose Tryptone (APT) agar are both widely used for cultivation and enumeration of LAB (deMan et al., 1960; Evans and Niven, 1951), special media have been developed which enable differential enumeration of these microorganisms.

2.2.4.1. MRSD medium

Although homofermentative lactobacilli and leuconostocs are major spoilage LAB found in vacuum-packaged cooked meat products, *Pediococcus* spp., especially when inoculated as starter cultures, can be present in significant numbers in sausages (Holley et al., 1988). It was observed that pediococci may dominate in sausages where glucono-delta-lactone was added in the formulation as an acidifier (Holley et al., 1988). The MRS differential medium (MRSD), used for differential enumeration of pediococci and lactobacilli (leuconostocs) was developed based on the fact that pediococci more readily utilize arginine when the glucose source is depleted (Holley and Millard, 1988).

It is well known that pedioccoci are capable of arginine hydrolysis (Sharpe et al., 1966). However, most heterofermentative lactobacilli and a few homofermentative lactobacilli such as *L. sake* are also able to utilize arginine in the absence of fermentable sugar (Schillinger and Lucke, 1986). By optimizing the formulation (with 1% (w/v) glucose and 0.1M L-arginine HCl with 0.0025% (w/v) phenol red as the pH indicator), the lactobacilli were discouraged from arginine utilization and the alkalinity generated reflected the presence of pediococci only (Holley and Millard, 1988). The hydrophobic grid membrane filter (HGMF) system was adopted to avoid the ambiguity generated by color diffusion from the colonies into the agar (Entis and Boleszczuk, 1986).

Like MRS medium, the MRSD medium only supports the growth of lactic acid bacteria. After one hour's staining with bromocresol purple, all the *Pediococcus* species appear blue and lactobacilli and leuconostocs appear green on the hydrophobic grid membrane (Holley and Millard, 1988).

2.2.4.2. M5 medium

M5 medium was derived by Zuniga et al., (1993) from the homofermentative-heterofermentative differential (HHD) medium developed by McDonald et al. (1987). The mechanism of differentiation was based on the fact that heterofermentative LAB reduce a portion of fructose to mannitol in addition to producing CO_2, lactic acid and acetic acid when fructose is the sole carbohydrate source. In contrast, homofermentative LAB only produce two mol of lactic acid from fermentable hexoses including fructose (McDonald et al., 1987). The larger amount of acid produced by homofermentative LAB causes a color change in bromocresol green, the pH indicator. In HHD broth tests, broth inoculated with homofermentative LAB was green while the sedimented cells were blue. Broth inoculated with heterofermentative LAB was blue while the cells were white (McDonald et al., 1987).

Zuniga et al. (1993) found that some LAB, including spoilage organisms, did not grow on HHD agar and some LAB generated aberrant results. By altering the composition of HHD medium (adding L-cysteine HCl, $MgSO_4$ and $MnSO_4$) to favor the growth of the spoilage LAB, they developed M5 agar. Zuniga et al. (1993) also pointed out that heterofermentative LAB can increase acid production under aerobic conditions because in the presence of O_2, fructose is not necessarily reduced to mannitol and a greater amount of acid can be produced and yield aberrant results (Condon, 1987; Zuniga et al., 1993). Incubation of M5 plates under strict anaerobic conditions is necessary to improve the use of fructose as an electron acceptor by heterofermenters.

Assays of the LAB strains indicated that all the homofermentative LAB formed blue colonies while heterofermentative strains produced white colonies on M5 agar. The time required to observe the test results was strain-dependent. Most strains grew in less than 7 days to give acceptable results (Zuniga et al., 1993).

On this medium, both *L. curvatus* and *L. sake* strains formed blue colonies and *Lc. mesenteroides* appeared white. This enabled direct isolation and differentiation of LAB from spoiled meats in my current research work.

2.2.5. Factors Affecting the Growth of Spoilage LAB

2.2.5.1. Temperatures

The LAB dominating in the spoilage of vacuum-packaged cured meat products are psychrotrophs. Therefore, they can grow reasonably well at low temperatures. Many strains of *L. curvatus* and *L. sake* are able to grow at 2-4°C (Kandler and Weiss, 1986). *Lc. mesenteroides* can grow at temperatures ranging from 10 to 37°C (Garvie, 1986).

The minimum, optimum and maximum growth temperatures of ropy slime-producing LAB able to spoil vacuum-packaged cooked meats were determined using a temperature-gradient incubator (Korkeala et al., 1990). It was found that the minimum growth temperatures of the lactobacilli strains were below -1 °C, while the minimum growth temperature of the spoilage *Lc. mesenteroides* strains was about 4 °C. It seems the lactobacilli can tolerate lower temperatures than leuconostocs. The optimum temperature for spoilage LAB is around 30 °C and the maximum growth temperature is below 40 °C. From the results, it also seems that the maximum growth temperature of lactobacilli is not a stable character because some thermo-resistant isolates were able to grow at a higher temperature than their parent strains (Korkeala et al., 1990). From the latter study it was shown that all the spoilage lactobacilli were able to multiply on MRS agar at temperatures below 0 °C. Therefore, low temperature storage itself can not totally prevent the eventual spoilage of vacuum-packaged cooked meat products.

2.2.5.2. pH values

It is well known that the spoilage LAB are able to produce lactic acid and/or acetic acid during the sugar fermentation, which is also a key advantage for these organisms, enabling them to dominate in meats by suppressing other competitors.

Lactobacilli are aciduric. The optimal pH usually ranges from 5.5 to 6.2. Growth generally occurs at 5.0 or less (Kandler and Weiss, 1986). For *Lc. mesenteroides* usually there is no growth at an initial pH of 4.8 but the final pH in glucose broth can reach as low as 4.5 (Garvie, 1986). It is difficult to find literature which reports the systematic investigation of different pH levels on the growth of spoilage LAB. During storage, the pH of cured meats may drop from 6.0-6.5 to 5.0 -5.3 due to the activity of LAB (Borch et al., 1996). Low pH values such as 4.6 can only be found when meats are unquestionably spoiled (Korkeala and Bjorkroth, 1997).

2.2.5.3. $NaNO_2$ concentrations

Lactic acid bacteria are quite resistant to nitrite although nitrite is quite effective against other bacteria such as *Clostridium botulinum* (Castellani and Niven, 1955). It was found that $NaNO_2$ had a very limited affect on the growth of *L. curvatus*, *L. sake* and *Lc. mesenteroides* at 50 and 100 ppm but there was an inhibitory effect at a 400 ppm (Korkeala et al., 1992). It was also found that leuconostocs were more sensitive to higher $NaNO_2$ concentrations than homofermentative lactobacilli. There was no significant difference between the *L. sake* and *L. curvatus* strains in response to the nitrite challenges (Korkeala et al., 1992).

2.2.5.4. NaCl concentrations

Lactic acid bacteria are able to grow at high NaCl concentrations. Most of the spoilage lactobacilli can survive 8% NaCl added to growth media (Reuter 1970; Mol et al., 1971). Another study showed leuconostocs were more sensitive to increasing NaCl levels than homofermentative lactobacilli and that *L. curvatus* strains were more sensitive to NaCl than *L. sake* (Korkeala et al., 1992). To inhibit the growth of spoilage LAB, considerable amounts of NaCl are needed, and these levels are greater than those normally considered organoleptically acceptable in cooked cured meats.

2.2.5.5. Bacteriocins

Bacteriocins were originally defined as naturally produced peptides that are antagonistic to other usually closely related bacteria. Interestingly, bacteriocins may play an important role in the ability of LAB strains to dominate in mixed populations (Klaenhammer, 1988). For example, among the bacteriocins produced by LAB strains a high percentage have antilisterial properties such as nisin (Carolissen-Mackay et al., 1997). Recently, bacteriocins have been further defined as " a heterogenous group of antibacterial compounds which are produced by a large and diverse group of bacterial species varying in their mode of action, activity spectrum, molecular weight, biochemical properties and genetic origin" (Klaenhammer, 1993). One major mechanism of bacteriocin action on target cells has been described as " membrane poration" (Muriana, 1996). Bacteriocin-producing LAB have recently received much attention because these organisms are major producers of these compounds and have a long history of safe use in foods either as starter cultures or as naturally occurring contaminants.

Agar spot and well diffusion tests can be used for qualitative study of bacteriocin production and resistance by using other LAB as indicator strains (Schillinger and Lucke, 1989). Treatments such as adjusting the pH, addition of catalase and proteases, and heat treatment (100 °C for 20-30 min) have been conducted to validate the presence of bacteriocins (Yang and Ray, 1994). Reports in the literature indicated that maximal bacteriocin production by *L. sake* strains occurred at pH 5.0 or lower, i.e., at pH values that would not be expected to occur in meats but may be attained in fermented meats. Negative effects of refrigeration temperatures (4-5°C) on bacteriocin production by meat LAB were also reported (Yang and Ray, 1994; Buncic et al., 1997). The feasibility of employing *in situ*-produced bacteriocin to prevent the spoilage of vacuum-packaged meats needs to be further investigated. However, *Listeria* inhibition by *in situ*-produced pediocin from a genetically manipulated *Pediococcus* strain during dry fermented sausage production has been reported (Foegeding et al., 1992).

2.2.5.6. Other factors

Other factors such as liquid smoke and sodium lactate may also be important parameters that affect the growth of spoilage LAB. Sodium lactate has been used in the meat industry for over 20 years, primarily for its humectant properties (Reid, 1969). A recent study showed that 3-4% sodium lactate had a significant inhibitory effect on *Leuconostoc* spp. after incubating the cooked beef at 0 °C for 56 days (Papadopoulos et al., 1991). However, an astringent taste caused by the high level of sodium lactate is still a problem to be solved.

Liquid smoke is a common ingredient in vacuum-packaged cured meat products. This material has been shown to have bactericidal action (Erdman et al., 1954). However, Donnelly et al. (1982) showed that levels of liquid smoke at twice the recommended use level (6 oz/100 lb.) had no significant inhibitory effect on the growth of LAB.

2.3. Pulsed-Field Gel Electrophoresis for *Lactobacillus* and *Leuconostoc* Strain Identification

2.3.1. Strain Characterization of Spoilage LAB

A diversity of LAB strains may be responsible for the spoilage of cured meats. In an investigation of dry fermented sausages, a total of 254 strains of lactobacilli was isolated from 15 different producers (Hugas et al., 1993). Biochemical characteristics such as sugar fermentation profiles can be used for the differentiation of strains but they usually do not allow complete identification. Many strains share the same biochemical properties and sometimes a strain will lose biochemical characteristics due to environmental influence. Spoilage LAB strains such as *L. curvatus* and *L. sake* strains are especially difficult to identify because these organisms can come from quite different environments and possess a diversity of biochemical and physiological properties (Kitchell and Shaw, 1975).

Since different LAB strains often dominate in different instances of meat spoilage, a variety of phenotypic and genotypic methods have been investigated for LAB strain identification. Dykes et al.(1995) studied LAB from processed spoiled cured meats using analysis of the cellular fatty acid composition patterns and found that this technique was able to distinguish these bacteria within but not between genera. Garver and Muriana (1993), and Holley et al.(1996) used the SDS-PAGE pattern of cell surface proteins to identify LAB but found it not practical for strain identification. Garriga et al. (1996) used electrophoretic patterns of plasmid DNA to identify *L. sake* strains which dominate during dry sausage production but it is not applicable to those LAB which do not contain plasmids. Bjorkroth et al. (1996) applied the techniques of randomly amplified polymorphic DNA (RAPD) and pulsed-field gel electrophoresis (PFGE) of restriction endonuclease-digested genomic DNA to characterize a large number of ropy and non-ropy slime producing *L. sake* strains. It was found that the PFGE of DNA digests had the greatest discriminatory power for strain identification (Bjorkroth et al., 1996)

2.3.2. PFGE Analysis of Spoilage LAB Strains

Pulsed-field gel electrophoresis (PFGE) consists of three unique techniques: intact genomic DNA purification, *in-situ* restriction endonuclease digestion and pulsed-field separation.

2.3.2.1. Theory of pulsed-field separation of large DNA molecules

Electrophoresis techniques developed in the last 60 years have allowed very effective separation of proteins and nucleic acids on the basis of molecular weight. However, conventional DNA electrophoresis is only effective to separate DNA fragments smaller than 20 kilo-base pairs (kb) long (Smith and Cantor, 1987). A piece of DNA 20 kb long only represents about 1% of the typical genome size of the LAB (Tanskanen et al., 1990), which means if we digest the genomic DNA into fragments smaller than 20 kb to set up the electrophoretic profiles, there will be hundreds of different bands on the gel and it will be difficult to interpret. Therefore, low frequency restriction endonucleases are introduced in the DNA digestion. These "rare cutters" usually recognize specific 6-base or 8-base sequences instead of 4 base sequences (Farber, 1996). After digestion one ends up with a small number (5 to 20) of very large and well-resolved fragments, typically ranging from 10 to 800 kb. The development of PFGE allows one to work with DNA fragments from 100 to 4×10^6 base pairs (bp), which generates a reduced number of electrophoretic bands (macrorestriction analysis) which are more manageable for comparative purpose.

2.3.2.1.1. Migration of DNA molecules in conventional electrophoresis

In conventional gel electrophoresis, charged molecules are sieved through an agarose or polyacrylamide matrix under a fixed electrical field. The larger molecules experience greater frictional resistance in spite the fact that they possess greater electrical charge. The net effect is that the small molecule moves faster than the large ones in the gel matrix. The gel matrix has a wide range of pore sizes. Small molecules will fit through most pores and thus can travel through the gel easily, but large molecules will be unable to enter most pores and will have to travel via a circuitous and much longer route (Smith and Cantor, 1987). Thus, the net translational velocity will be much smaller. Agarose is chosen for DNA fragments above the size of 500 bp because it has large pores. Polyacrylamide is used for the size range of one to 700 bp because it has a smaller pore size (Smith and Cantor, 1987).

Usually it is not possible for DNA fragments larger than 20-25 kb to be separated by conventional gel electrophoresis because the molecules are larger than the pore sizes (Farber, 1996). However, it is known that the double-stranded DNA molecules are spherical Gaussian coils. Therefore they can travel through the agarose gel matrix by deforming their shape in order to pass through the pores (Figure 3, a). Under a constant electrical field, the molecules always travel along their axis like the movement of snakes (Levene, 1992). Because the frictional pull exerted per unit length of DNA is predetermined and almost evenly distributed when the long molecules go through the

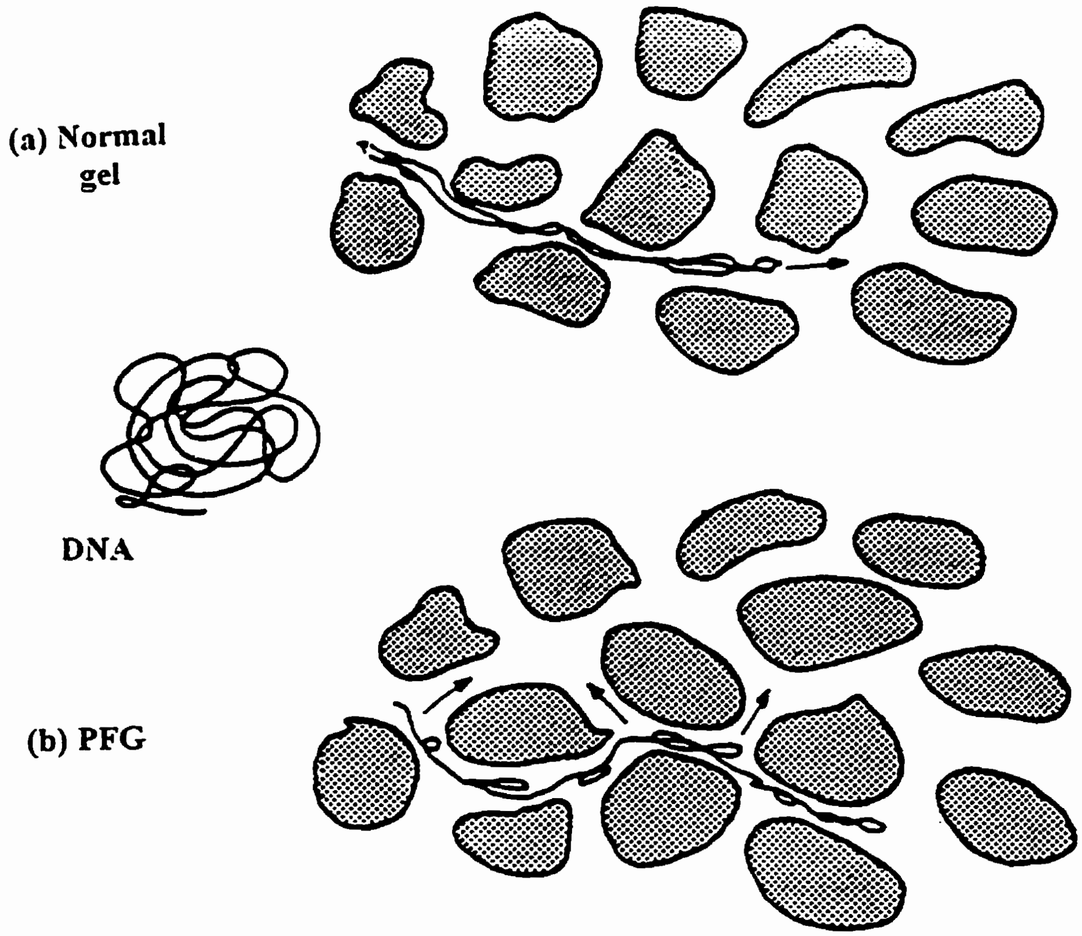
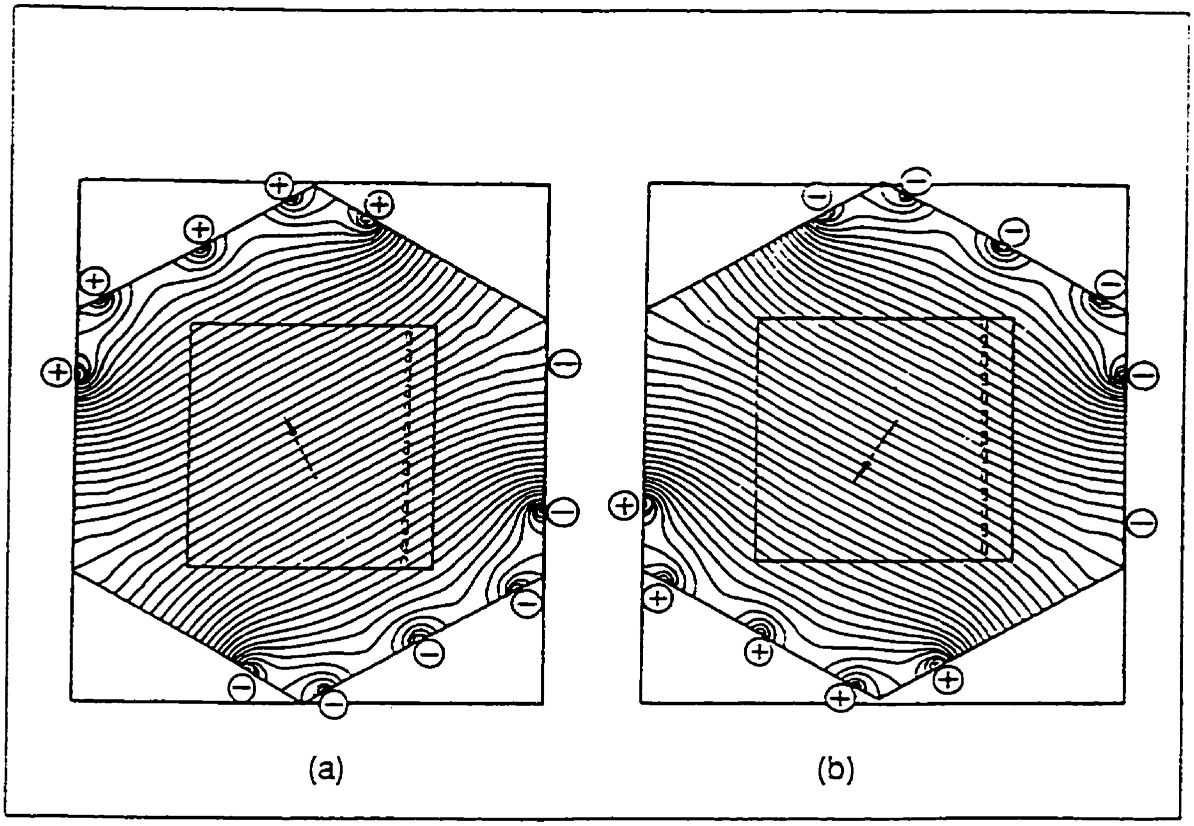


Figure 3. Separation of DNA by ordinary and pulsed- field gel electrophoresis. (Smith and Cantor, 1987). DNA molecule traveling under standard electrophoresis is oriented parallel to the field (a). DNA traveling under pulsed-field is forced to spend most of its time reorienting itself perpendicular to its long axis (b).

pores, the velocity of the fragments will be independent of size, ie., all the large molecules have the same speed along the field direction and no fractionation can be achieved (Smith and Cantor, 1987).

2.3.2.1.2. Migration of DNA molecules in PFGE

In the PFGE technique, especially some newly developed revisions, a homogenous electrical field is applied alternately in two directions at an angle of 120° with the hexagonally arranged electrodes (Figure 4). It is also referred to as "pulsed field". Changes in field direction force DNA molecules to alternately orient themselves in two directions. Molecules traveling along the DNA axis have to deform themselves to fit in another field direction applied at 120° to the original axis. The field changes again so the DNA molecules are forced to adjust themselves to a new field orientation and travel in a winding way under this pulsed electrical field (Figure 3, b). The time spent in each field direction is called pulse duration or pulse time. If the pulse duration is too long, there will be more than enough time for the DNA molecules to reorient themselves and then travel in the manner of conventional electrophoresis. Therefore, no size fractionation will occur. If the pulse duration is too short, the molecules will have no time for reorientation and find themselves traveling under a net field with the direction between the two alternating electrical fields. Again there will be a conventional electrophoresis and no separation can be achieved (Smith and Cantor, 1987). However, at intermediate pulse duration, DNA fragments will spend most of their time reorienting and effective size fractionation will occur because the time allowing one molecule to reorient itself in the gel matrix from one



(a)

(b)

Figure 4. Two different fields are applied in the Gene Navigator system, using the hexagonal electrode arrangement. (User Manual, Gene Navigator System, Pharmacia Biotech AB, Uppsala, Sweden.) The figures show isofield lines and the arrows show the field direction. (a) North/ South field; (b) East/ West field.

field direction to the other depends on its size (Figure 5).

The pulse duration usually ranges from 0.1 second to 200 minutes depending on the size of the DNA fragments. The run time, i.e., the time of the alternating field application in a certain pulse duration pattern can be several hours (Meng et al., 1995). Generally the separation of larger DNA molecules needs both a longer pulse duration and longer run time. This is because at long pulse durations, short molecules complete their reorientation very fast and will then move by conventional electrophoresis. Since longer molecules move with lower velocity, a longer run time is necessary to achieve satisfactory resolution. For a set of DNA digests with a range of molecular weights (1-250 kb), usually a series of pulse durations and run times will be programmed continuously at one time to achieve effective separation of DNA bands in different size ranges. For example, one program can be: 0.3 second pulses for one hour; 0.5 second for one hour; 0.7 second for one hour; 2 seconds for 5 hours and 4 seconds for 6 hours (Bjorkroth et al., 1996).

A quantitative understanding of the effects of rapid field changes on the mobility of DNA molecules remains largely incomplete due to the relative simplicity of the models used to describe gel electrophoresis. There are several different statistical-mechanical theories for the gel electrophoresis of DNA and their application to the analysis of PFGE experiments (Levene, 1992). These will not be further discussed here.

With the PFGE technique, genomic DNAs of *Lactobacillus* and *Leuconostoc* strains digested by *Sma*I can be clearly mapped on agarose gels (Bjorkroth et al., 1996; Kelly et al., 1995). Even intact chromosomal DNAs of yeast and the malaria parasite *Plasmodium falciparum* which are several million bp long, can be successfully separated

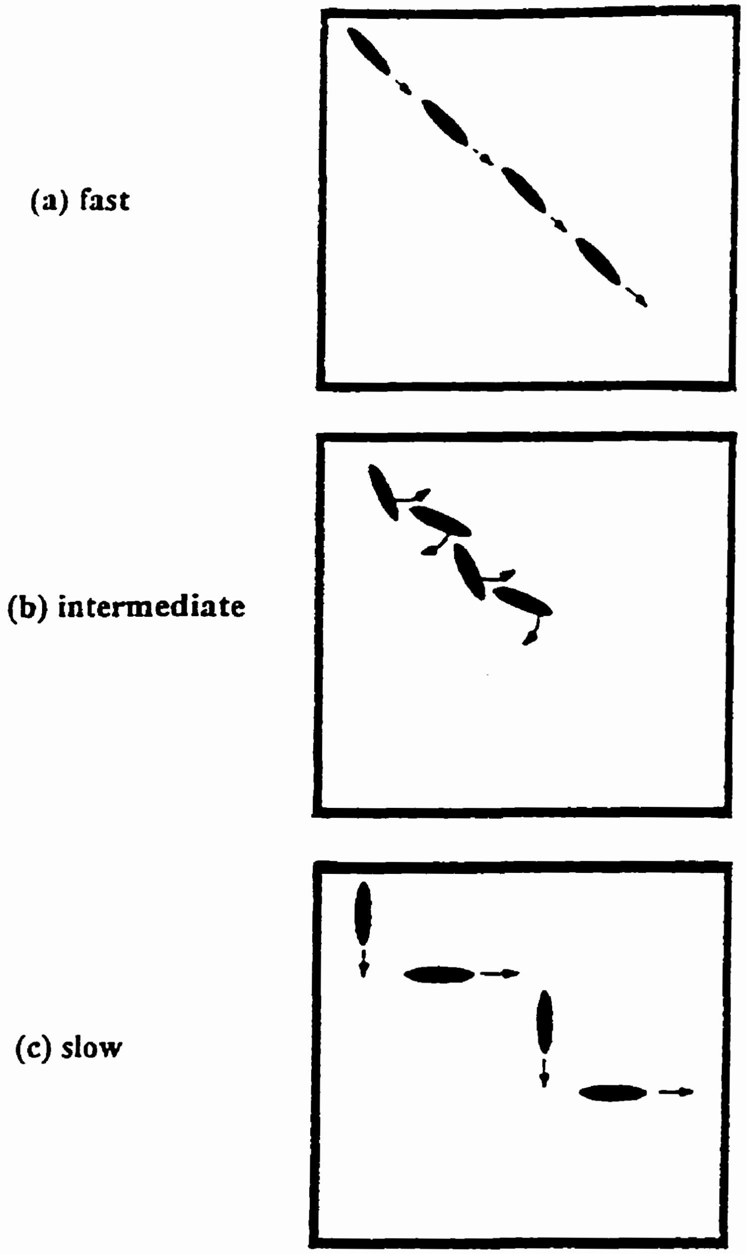


Figure 5. The effect of pulse durations on the separation of DNA molecules. (Smith and Cantor, 1987). When the pulse duration is too short, the molecules travel straight under the average electric field and yield results similar to conventional electrophoresis (a). When the pulse duration is too long, the DNA molecules completely reorient themselves and then travel along their axis by conventional electrophoresis, still no separation can be achieved (c). At intermediate pulse duration the molecules spend most of their time reorienting themselves, therefore successful fractionation occurs (b).

using this technique (Kemp et al., 1985).

2.3.2.2. Purification of intact bacterial genome

Conventional DNA preparative procedures are carried out in solution. If the cells are intact, the cell suspensions will be first treated with appropriate enzymes to remove the walls. The resulting spheroplasts or protoplasts are then broken with detergents and EDTA, a metal chelator. This produces a complex mass of proteins, DNA and RNA (Smith and Cantor, 1987). RNase is added to get rid of the RNA which may affect the resolution of the final electrophoresis patterns. Proteins are degraded by proteases. Usually these procedures will be repeated several times to get clean DNA preparations. It is also very important that in each step the samples are not exposed to DNases except for the endogenous ones.

Further manipulation usually involves washing samples by centrifugation and transferring by pipetting. These handling methods always break DNA molecules into random pieces less than 10^6 bp long. This is acceptable for ordinary preparations because subsequent standard molecular techniques usually involve working with DNA fragments less than 20 kb long (Smith and Cantor, 1987). However, it's critical for the PFGE to get essentially intact genomic DNA.

In order to work with intact DNA molecules, one must prevent the shear damage that occurs readily in solution procedures. Recently a new and simple technique has been developed which involves purifying genomic DNA from freshly grown cells held in solid agarose blocks. These latter are also called “inserts”or “plugs”(Smith and Cantor, 1987).

In the inserts, the DNA is protected from shearing damage because the long fragile DNA molecules are immobilized in the agarose gel matrix upon its release from the cell. It is very easy to handle in this state without mechanical damage. Small molecules can move in and out of the gel matrix freely so the *in situ*-DNA purification and digestion are feasible.

In the PFGE analysis of *Lactobacillus* and *Leuconostoc* strains, suspensions of *L. curvatus*, *L. sake* and *Lc. mesenteroides* strains at a concentration of ~10^9 CFU/ml are incorporated in 1% low melting point agarose gel. There are three reasons for choosing low melting point agarose as the fixing material. The low melting point agarose has a melting point of 62.5 °C and a gelling point of about 28°C so it is easy to handle without the need for great haste and the heat denaturation of the cell components before enzyme treatment can be avoided. The third reason is that its fine structure allows free diffusion of reagents and wastes. Typically the inserts are 2×5×10 mm and each insert contains about 10^8 cells which is equal to about 10 µg of DNA (Smith and Cantor, 1987). Since 1-2 µg is enough for one electrophoresis run, one insert can be divided for 5-10 runs.

Once cells are immobilized, the gel inserts are incubated in lysis buffer which is used to destroy the cell wall and then incubated in ESP buffer (Chapter 3). Intensive washes are conducted to obtain very clean intact DNA. The ESP buffer is a powerful reagent which removes cell proteins. In some PFGE protocols the lysis procedure is even omitted and cell inserts are only treated with ESP buffer (Farber, 1996).

2.3.2.3. Restriction endonuclease digestion of intact DNA molecules

Although the intact chromosomes of bacteria are only 0.75-20 Mb, they do not migrate in any electrophoresis system due to their circular topology(Smith and Cantor, 1987). Therefore, the fragmentation of the big DNA molecules into a convenient number of linear DNA pieces is a necessary prerequisite for analysis of the bacterial genome. Rare cutting restriction endonucleases, which cut DNA infrequently at specific sequences are used to generate DNA fragments in the optimal size range for PFGE . Most restriction endonucleases have 4- or 6-bp recognition sequences. For example *Sma*I will recognize 5'-CCC↓GGG-3' and cut between C and G. For the lactobacilli and leuconostocs, G+C represents around 43%(≤50%) of the total genome bases (Kandler and Weiss, 1986), which means an endonuclease recognizing only GC sequences such as *Sma*I will theoretically cut the DNA more infrequently than other restriction enzymes (Tanskenen et al., 1991). This is the reason why *Sma*I is widely used for the PFGE analysis of these organisms. Simple statistics shows that, if the four different bases are randomly distributed in the DNA chain, *Sma*I will cut the DNA at every 4^6 (4096) bases (Farber, 1996). That means about 500 bands will be generated. However only 5-20 bands can be created since the four bases are organized in the DNA chain according to the genetic nature of the organism. If the restriction enzyme *Not*I which recognizes 8-base sequences is used, less than 10 bands will appear on the gel (Kelly et al., 1993). Sometimes it is easier to interpret results with fewer bands. When a single enzyme is unable to differentiate all the strains in a test group, other enzymes may be applied and satisfactory results can be obtained by combined interpretation (Bjorkroth et al., 1996). In fact, many studies have shown that bacterial strains which cannot be distinguished by PFGE are not likely to show substantial differences when tested by other typing methods (Tenover et al., 1995).

To continue the protocol, the inserts stored in ESP buffer are cut into 1×2×5 mm slices. Slices are washed in TE buffer (Chapter 3) containing 0.1 mM phenylmethylsulfonyl fluoride(PMSF). PMSF is a protease inhibitor (Farber, 1996). It is used to inactivate proteinase K and prevent interference with the subsequent endonuclease digestion . Slices are then washed in endonuclease buffer. Intense wash steps help to get very clean gel slices and ensure the optimal activity of the enzyme in buffer. A restriction endonuclease such as *Sma*I is added and the samples are incubated at appropriate temperatures for the specific enzymes. The digested DNA samples then can be washed in ES and ESP buffer (Chapter 3)again and stored at room temperature for several months.

2.3.2.4. PFGE separation procedure

The slices now can be loaded into the running gel slot. The running gel is made from 1.2-1.5% (w/v) agarose dissolved in 0.5 ×TBE buffer (Chapter 3). Samples are electrophoresed through the gel for a programmed time at 200V and 14°C in a PFGE system with a hexagonal electrode. After electrophoresis the gel is stained with ethidium bromide and visualized under UV light. Ethidium bromide is used as the stain because it can intercalate between the bases of nucleic acids and flouresces when it is exposed to UV light. It is widely used to resolve the fragments for photography and it should be handled with caution because it is a carcinogen (Tenover et al., 1995).

2.3.3. Summary

Pulsed-field gel electrophoresis is one of the key advances in the past 15 years in molecular biology(Levene, 1992). It can separate very large DNA molecules in an agarose gel that cannot be separated by conventional methods. Separation is based on the fact that the reorientation time for differently sized DNA molecules under the alternating electrical field is different. By applying appropriate pulse durations and run times, successful fractionation of a range of DNA with different sizes can be achieved.

The PFGE technique also involves the *in-situ* purification and digestion of intact DNAs which is quite different from that in standard molecular methods. The purpose of PFGE analysis is to distinguish individual strains of microorganisms by their macrocleavage patterns. That is to say, any breakdown of the DNA molecules other than by restriction endonuclease digestion is undesirable and will yield false information. Therefore, purification and restriction analysis of intact genomic DNAs are carried out in low melting point agarose gel inserts which will not cause undue physical shearing to the DNA molecules. The infrequent cutting restriction endonucleases are chosen for the analysis because these "rare cutters" can generate a convenient number of fragments. An endonuclease is considered powerful if it is able to create a maximum number of band differences among the bacterial strains under test.

A variety of studies have indicated that effective strain differentiation can be achieved by PFGE analysis. This technique is becoming a powerful tool for characterization of strains of spoilage organisms and also for the epidemiological study of food pathogens. In the battle with cured meat spoilage, before a proper inhibitory system can be designed, it is important to understand factors which influence dominance of spoilage strains among the background microflora present. Varying growth conditions in laboratory tests will be used in conjunction with testing in special meat formulations to obtain a better understanding of reasons for the dominance of specific organisms and their ability to compete successfully in package environments. PFGE can be used as a powerful tool to trace the growth of single organisms in mixtures where bacteria are phenotypically (biochemically) similar.

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

3.1.1. Meat Samples

Spoiled vacuum-packaged sliced ham samples (Smoked Pepper Loaf) which showed the presence of purge, were contained in a loose or "blown" package or contained slime were obtained from a local supermarket. Spoiled vacuum-packaged corned beef samples exhibiting slime were supplied by an Ontario meat manufacturer.

3.1.2. Bacterial Strains

Strains No.1, 3, 5, 6, 9, 10, 11, 13, 14 and 15 were isolated from retail meats (Holley et al., 1996). Strains C, R, CBLb1, CBLb2, CBLb3, CBLc1 and CBLc2 were isolated from spoiled commercial meat samples in this study. Strain No.23 was from the DSM culture collection, Germany. Strains No.33 (ATCC 23386), No.34 (ATCC 15521) and No.35 (ATCC 25601) were purchased from American Type Culture Collection (Rockville, MD).

Among the strains which were used in this study, No.3, 5, 10, 15, 23, 35 and R were *L. curvatus;* No.6, 9, 14, 34, CBLb1, CBLb2 and CBLb3 were *L. sake;* No.1, 11, 13, 33, C, CBLc1 and CBLc2 were *Lc. mesenteroides.*

3.1.3. Chemicals (Appendix 1)

3.1.4. Media and Reagents (Appendix 2)

3.1.5. Supplies and Instruments (Appendix 3)

3.2. Methods

3.2.1. Strain Isolation

Spoiled meat samples were collected from a local supermarket and a national manufacturer. For vacuum-packaged sliced ham, 11 g of meat were taken aseptically from the surface slices. When whole pieces of meat such as corned beef were analyzed, a stainless steel core device and a knife were employed to cut out 11 g (about 30 cm^2) of surface meat with 2-3 mm in thickness (avoiding fat). For the samples possessing slime, the slime was collected and analyzed separately. The information on the meat package was recorded for later reference. The meat was then mixed with 99 ml peptone water (0.1% trypticase peptone) in a stomacher bag and massaged for about 1 min (Stomacher lab-blender, model 400, Seward Laboratory, London, UK). Decimal dilutions were made using 99 ml dilution bottles with peptone water. Dilutions were plated on M5 agar plates using a surface spread-plating technique and incubated anaerobically at 25°C for 48 h.

For MRSD agar plates, the hydrophobic grid membrane filter (HGMF) technique was used. A sterile HGMF filter (QA Labs Ltd., Toronto) was placed in a sterile HGMF filtration tower and was fixed using a clamp. Thirty to 40 ml of peptone water was flushed down from the top of the tower to rinse the metal prefilter and the membrane filter. One ml of the sample dilution was pipetted into the peptone water in the top opening of the tower. A vacuum was applied to draw the remaining peptone water from the top into the small chamber of the filtration tower. Some more peptone water was used to flush the metal wire net of the tower and a vacuum was applied later to remove all the liquid from the membrane filter. Then the membrane was transferred aseptically onto the surface of a pre-dried MRSD agar plate. After incubation, the filter membrane was post-stained with 0.4% (w/v) bromoscresol purple to show color differences in colonies.

Plates were incubated anaerobically in jars using GasPak® CO_2 atmosphere anaerobic system with an anaerobic indicator at 25°C for 48 hours. Plates with an appropriate number of colonies (30-300) were counted using a dark field colony counter.

3.2.2. Strain Identification

Colonies on M5 and MRSD were examined by color and transferred into 50 ml MRS broth. The flasks were labeled and incubated at 25°C overnight. Each isolate was subcultured twice and preserved at - 80°C following standard procedure (Appendix 4).

3.2.2.1. Gram test

The Gram test was performed according to Gregersen (1978). One drop of 3% KOH was placed on a piece of microscope slide. One colony was picked up using a platinum loop and mixed with the KOH solution. After several minutes, the mixture was examined using the loop to check for ropiness. A positive result (ropiness) indicated that the organism was Gram negative.

3.2.2.2. Catalase test

One drop of 3% H_2O_2 was mixed with the colony under examination on a microscope slide using a platinum loop. Gas (O_2) production on the slide was recorded as a positive result for catalase (Harrigan and McCance, 1976).

3.2.2.3. CO_2 production test

One liter of CO_2 production test broth was prepared according to the formula and brought to a boil to dissolve completely. Ten milliliters of broth were dispensed in each test tube containing inverted Durham vials. After autoclaving and cooling, tubes were inoculated with the pure culture under examination and incubated at 25°C anaerobically for 2 days. Gas trapped at the top of Durham vials represented a positive result (Schillinger and Lucke, 1987).

3.2.2.4. Arginine utilization test

One liter of arginine hydrolysis test broth was prepared according the standard formulation (Schillinger and Lucke, 1987). The broth was dispensed 10 ml per test tube (18x150 mm). After sterilization by autoclaving, the broth tubes were inoculated with pure culture and incubated at 25°C anaerobically. The result was examined after 3, 5 and 7 days respectively. For the examination, 2 drops of culture supernatant and 2 drops of Nessler's reagent were mixed on a microscope slide and a color change from yellow to orange was concluded to be a positive result.

3.2.2.5. Sugar fermentation tests

MRS broth without glucose and beef extract was prepared and dispensed 10 ml per tube (18x150 mm) with screw caps and autoclaved. Test sugars (0.5% of each of: trehalose, melibiose, maltose, arabinose and amygdalin separately) plus 0.01% bromocresol green were filter sterilized and added to the broth. Tubes were inoculated with test strains and incubated anaerobically at 25°C. The tubes were examined after 2 and 4 days. A color change of the broth from blue to yellow was regarded as positive (Schillinger and Lucke).

3.2.2.6. API 50 CHL test

The cultures being tested were grown in MRS broth up to the logarithmic growth phase. The cells were then washed twice in sterile saline by centrifugation at 10,000 x g for 20 min. The cell suspension was spectrophotometrically calibrated between 0.5 and 0.7 using the McFarland standards (Finegold and Martin, 1982) and inoculated into the API 50 CHL medium according to the commercial instruction (bioMerieux SA, Marcy-l'Etoile, France). The inoculated medium was then distributed into the 50 tubes on the test strips. Anaerobic conditions were created by overlaying all the tubes with sterile mineral oil. The prepared trays containing the test strips were incubated at 30°C for 48 hours. The ability of the strains to use the carbon sources was determined on the basis of the color change in bromocresol purple, from purple to yellow. For the esculin test (tube No.25), a change from purple to black was observed in positive reactions. Readings were taken after 3,6, 24 and 48 hours. After the fermentation profiles were determined, the strains were identified to species using the commercially provided identification table.

3.2.2.7. Pulsed-field gel electrophoresis (PFGE) analysis of SmaI digested genomic DNA

3.2.2.7.1. Genomic DNA *in situ* purification

For PFGE analysis of *Lactobacillus* and *Leuconostoc* strains, the *L. curvatus*, *L. sake* and *Lc. mesenteroides* cultures were incubated in MRS broth at 27°C for 48 hours.

Cells were washed in 50 mM Na_2EDTA (pH 7.5) buffer by centrifugation and resuspended in the same solution. Two ml of cell suspension and an equal volume of 2%(w/v) low melting point agarose gel were equilibrated to 42°C and mixed together. The mixture was pipetted into the insert molds and allowed to set at 5°C. Typically the inserts were 2×5×10 mm in dimension and one insert could be sliced and provide enough material for 5-10 runs.

Then the gel inserts were transferred to sterile empty glass test tubes (18x150 mm). A lysis buffer (pH7.6) containing 6 mM TrisHCl, 100 mM Na_2EDTA, 1 M NaCl, 0.5% sarkosyl, 10 U/ml mutanolysin, 1 mg/ml lysozyme and 20 µg/ml RNase was added to the gel inserts. Inserts were incubated at 50°C overnight with gentle shaking using a controlled environment incubator shaker (Lab-Line Instruments, Inc., Melrose Park, IL) and the lysis was repeated again at 37°C. Transferring of liquid into and out of the tubes was done by sterile syringes fitted with needles.

After lysis the waste buffer was poured off and 3 ml of ESP buffer (containing 0.25 mM Na_2EDTA, 1% sarcosine and 0.5 mg/ml proteinase K) were added. Tubes were incubated for 2 days at 37°C. After incubation the finally purified intact genomic DNA sample can be stored at 4°C with the ESP buffer indefinitely.

3.2.2.7.2. Restriction endonuclease digestion of intact DNA molecules

The inserts stored in the ESP buffer were cut into 0.5×2×5 mm slices. This step was performed on ice to get symmetric slices using sterile razor blades. Slices were transferred to sterile Eppendorf tubes. Slices were washed in TE buffer (10 mM TrisHCl and 1 mM Na_2EDTA) containing 0.1 mM phenylmethylsulfonyl fluoride(PMSF) for 12 hours at 25°C and the wash was repeated once.

Slices were again washed in TE buffer at 25°C for 2 hours and the wash was repeated twice. Then the slices were washed in 0.5 ml endonuclease buffer (450 µl sterile deionized water, 50 µl restriction endonuclease 10 ×buffer and 5 µl acetylated bovine serum albumin) at 25°C for 15 minutes and this was repeated twice.

Ten to 20 units of *Sma*I were added to each tube and tubes were incubated overnight at 30°C. Slices were then washed in ES buffer (ESP buffer without proteinase K) for 2 hours at 50°C and then in ESP buffer for 2 hours at 50°C.

3.2.2.7.3. PFGE separation procedure

About 2.5 liters of 0.5 ×TBE buffer (45 mM TrisHCl, 4.5mM boric acid, pH8.3 and 1 mM Na_2EDTA) were prepared and poured into the buffer tank of the Gene Navigator equipment. The refrigerated water bath and the pump on the buffer tank were turned on to pre-chill the buffer. The water bath was set at 9°C so that the temperature in the electrophoresis unit (buffer tank) remained at 14°C.

About 1.65 grams of agarose specially made for PFGE were added to 110 ml of sterile 0.5 ×TBE buffer and were dissolved by gentle heating in a 90°C water bath. When the gel was cooled to about 70°C, it was poured into the rubber frame sitting on the gel-supporting tray. A 1 mm thick plastic comb with 25 teeth was inserted in the agarose and arranged to sit towards one side of the gel. After 30 minutes, the rubber frame was removed and the comb was gently pulled out first by one side then the other. The digested sample slices and one slice of size standards (Pulse marker, 0.1-200 kb, Sigma)were loaded into wells of the running gel. The wells were then sealed with 42°C low melting point agarose gel (1%(w/v)).

The gel supporting tray holding the gel slab was then immersed in the electrophoresis buffer in the tank. The samples were electrophoresed through the gel following a programmed pattern (pulse duration at 0.3 second for 1.5 hours; 0.5 second for 1.5 hours; 0.8 second for 2.5 hours; 2 seconds for 3.5 hours; 5 seconds for 4 hours and 10 seconds for 5 hours) which was generated and stored in the GN controller. The program was run at 200V in the Gene Navigator system equipped with a hexagonal electrode. After electrophoresis the gel was stained in 0.5 µg/ml ethidium bromide solution for 30 minutes and then visualized under UV light through a Gel Doc 1000 system or photographed using Polaroid 667 film using a Polaroid MP4 Land camera.

3.2.3. Test of Bacteriocin Production and Resistance

3.2.3.1. Agar spot test for bacteriocin screening

In this test, 0.5 µl of cultures grown at 25°C for 18 h were spotted in an equidistant manner onto MRS agar plates (with surface pre-dried) and incubated overnight at 25°C. Four strains were tested in one plate. Plates were then overlaid with 5 ml of MRS soft agar

(0.7% agar) inoculated with 20 µl of the potential indicator strain (Schillinger and Lucke, 1989). The soft agar contained 1% morpholinoethane sulphonic acid (MES) to act as a buffer agent and the pH was adjusted to 5.4 before autoclaving (Buncic et al., 1997). Plates were examined after 24 h incubation at 25°C for the presence of colonies surrounded by clear inhibition zones. Twenty isolates used as both the testing and indicator strains included those lactobacilli and leuconostocs isolated from spoiled meat in this study in addition to a range of other LAB strains isolated from retail meats (Holley et al., 1996)

3.2.3.2. Well diffusion test

Strains showing positive results in the agar spot assay were grown in 50 ml MRS broth in flasks at 25°C for 48 hours. After centrifugation of the culture broth, the supernatant was transferred to 50 ml beakers and subjected to several assays.

Plates containing 25 ml of MRS agar inoculated with ~10^7 CFU/ml indicator strain were prepared. Seven wells were made aseptically using a cork borer, one in the center and six at the periphery. The well bottom was sealed with soft agar. Fifty µl of original testing strain supernatant were filter-sterilized and transferred to the center well. Equal amounts of supernatant, treated by: (1) adjusting the pH to 6.5; (2) making a 10 fold concentration by freeze drying; (3)addition of 1 mg/ml catalase from bovine liver; (4) addition of 1 mg/ml proteinase K; (5) addition of 1 mg/ml protease; (6) addition of 1 mg/ml trypsin, separately, were filter-sterilized and transferred to the peripheral wells (Kelly et al., 1993). Plates were incubated at 25°C for 48 hours. Positive results were recorded when observation of clear inhibition zones was made around the wells.

3.2.3.3. Modified agar spot test for bacteriocin production confirmation

Strains showing positive results in the agar spot assay were grown in 50 ml MRS broth in flasks at 25°C for 24 h. After incubation, 0.5 µl of cultures were spotted onto MRS agar plates and incubated overnight. Five spots of the same strain were arranged in one plate. Five µl of filter-sterilized catalase (1 mg/ml), proteinase K (1 mg/ml), protease (1 mg/ml) and trypsin (1 µg/ml) were spotted, respectively, beside 4 of the 5 developed colonies (Buncic et al., 1997). The remaining colony was left untreated as control. Plates were placed right side up at room temperature for 2 h to allow complete diffusion of the reagents. Plates were then overlaid with the indicator strain (in soft agar containing 1% MES) and incubated overnight at 25°C. Positive results were recorded when clear inhibition zones around the colonies with asymmetric outlines were observed.

3.2.4. Individual Inhibitory Factor Study

3.2.4.1. Strain selection

A modified MRS (MMRS) broth (containing 2.5% NaCl, 100 ppm $NaNO_2$, 1% glucose, pH 6.0 to mimic a standard cured meat formulation) was prepared and 10 ml were dispensed into 18x150 mm test tubes. After autoclaving, 11 *Lactobacillus* and 4 *Leuconostoc* strains in MRS broth were inoculated into test tubes containing MMRS broth to yield 10^7 CFU/ml. Tubes were incubated at 6°C anaerobically for 2 weeks. After incubation, the bacterial levels were quantified by spread plating on MRS agar plates. A total number of 6 strains, 2 from each species which showed the most exuberant growth were selected for further study.

3.2.4.2. Individual factor test

Four factors: pH, temperature (combined with incubation time), NaCl concentration and $NaNO_2$ concentration were tested individually for their inhibitory effects on test organisms. For each factor tested, duplicates were made. Final pH values of the broth were measured and serial dilutions were plated on MRS agar for enumeration. All tests were repeated twice.

3.2.4.2.1. pH values

Standard MMRS broth (2.5% NaCl, 100 ppm $NaNO_2$) was prepared and divided into 3 portions. Then pH values were adjusted to 5.5, 6.0 or 6.5. Ten ml of broth were dispensed in 18x150 mm test tubes. After sterilization, the tubes were inoculated separately to yield 10^7 CFU/ml of each of the 6 strains chosen in the previous test and incubated at 6°C anaerobically for 14 days.

3.2.4.2.2. Temperatures

Standard MMRS broth was made and dispensed in tubes. Tubes were evenly divided into 3 groups. Bacterial strains were inoculated as described before. One group was incubated anaerobically at 12°C for 7 days to represent abusive product storage conditions. Another group was incubated anaerobically at 6 °C for 14 days to represent the normal commercial conditions at retail. The remaining tubes were incubated anaerobically at 2°C for 35 days to mimic more desirable storage conditions.

3.2.4.2.3. NaCl concentrations

Standard MMRS broth without NaCl was prepared and divided into 3 equal parts. NaCl was added to the broth to yield 3 levels: 2.5%, 4.0% and 5.5% (w/v). After sterilization and inoculation as described before, test tubes were incubated anaerobically at 6°C for 14 days.

3.2.4.2.4. $NaNO_2$ concentrations

Standard MMRS broth without $NaNO_2$ was prepared and divided into 3 equal volumes. $NaNO_2$ was added to the broth to yield 4 levels: 50 ppm, 100 ppm, 150 ppm and 200 ppm. After sterilization and inoculation as described before, test tubes were incubated anaerobically at 6°C for 14 days.

3.2.5. Dominance Test

Based on their growth characteristics as well as their ability to produce or resist bacteriocin, strains No.1, 9 and 10 were chosen for the dominance tests. In these experiments, strain No.1 (*Lc. mesenteroides*) was paired with either strain No.9 (*L. sake*) or strain No.10 (*L. curvatus*) in 3 different ratios: 10^7:10^4, 10^4:10^4, 10^4:10^7 (CFU/ml), respectively.

In order to obtain an accurate initial cell concentration for this test, the relationship between bacterial count and optical density for each strain was studied. Cultures were grown in MRS broth for 48 hours at 25°C to reach the stationary phase. The broth was centrifuged and the cell pellet was re-suspended in 0.85% sterile saline. This was repeated

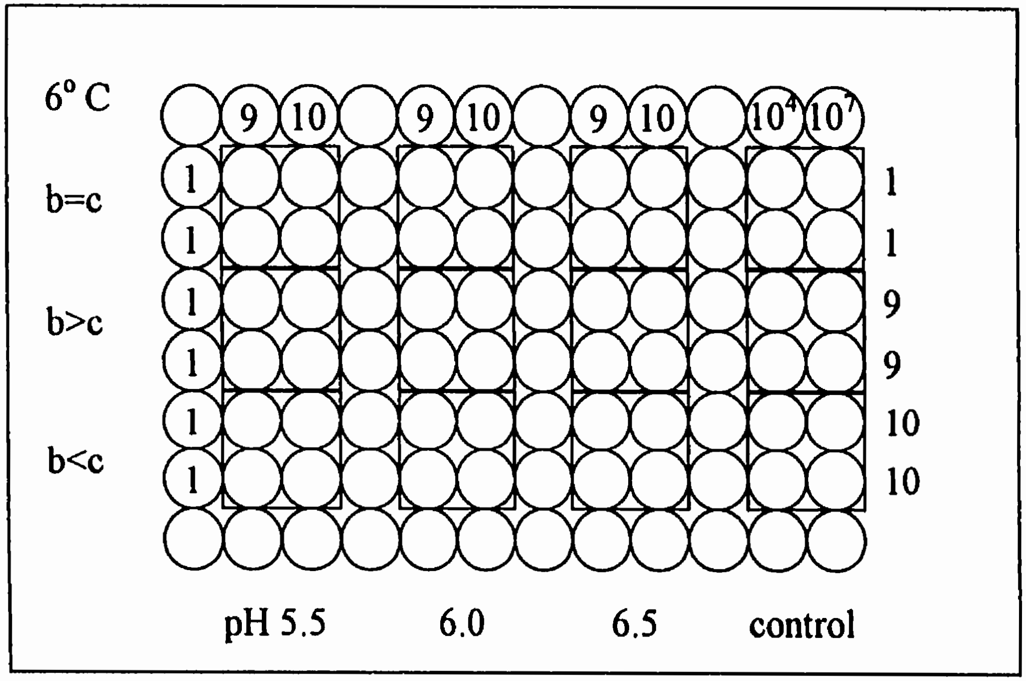


Figure 6. The arrangement of a 96-well microtitre plate incubated at 6°C for 14 days. The column labeled with "control" on the right consisted of 12 wells containing standard MMRS broth inoculated with each organism as a control. In the two rows (12 wells) labeled with "b = c", the initial number of both *Lactobacillus* and *Leuconostoc* was 1×10^4 CFU/ml. In the two rows labeled with "b > c", the ratio of *Lactobacillus* vs. *Leuconostoc* was $1 \times 10^7 : 1 \times 10^4$ (CFU/ml). In the two rows labeled with "b < c", the ratio of *Lactobacillus* vs. *Leuconostoc* was $1 \times 10^4 : 1 \times 10^7$ (CFU/ml). The pH of MMRS broth in Columns 2&3, 5&6 and 8&9 were 5.5, 6.0 and 6.5, respectively.

once. Serial dilutions were made and for each dilution, the optical density was measured and the cell suspension was plated on MRS agar for enumeration. For the measurement of optical density, 3 ml cell suspensions were placed in 1x1x4.5 cm standard disposable cuvettes and optical density was measured at $OD_{550\,nm}$ (Gill et al., 1995) using an Ultraspec 2000 UV/visible spectrophotometer (Pharmacia Biotech AB, Uppsala, Sweden). A linear relationship between the cell concentrations and optical densities was obtained around 10^6 -10^8 CFU/ml levels and the optical densities for $4x10^7$ CFU/ml cell suspension were plotted for each strain (1,9 and 10). Bacterial suspensions of $4x10^4$ CFU/ml were obtained by 1000 fold dilution of the $4x10^7$ CFU/ml culture.

In this experiment, double-strength MMRS broth with 3 pH levels (5.5, 6.0, 6.5) was prepared and 100 µl of broth were pipetted into wells in microtitre plates (96 well Falcon 3072 Microtest III tissue culture plate, Becton Dickinson & Co. Lincoln Park, NJ) using a 1-100 µl Eppendorf Reference pipetter. Then the paired strain suspensions, 50 µl each, with specific concentrations ($4x10^7$ or $4x10^4$ CFU/ml) were pipetted into the wells with double strength MMRS broth to give a 200 µl final volume of standard MMRS broth. The arrangement of wells in the microtitre plates for this test is illustrated in Figure 6.

To avoid foaming and evaporation during vacuum-packaging and condensation during incubation, one microtitre plate filled with sterile water was put on top of the plates being vacuum-packaged and these plates were vacuum-packaged together using high O_2 barrier pouches (Deli 1, Winpak) and a Bizerba GM 2002 vacuum packager (Mississauga, Canada). Three sets of plates with mixtures replicated twice, were then incubated at 12°C for 7 days, 6°C for 14 days or 2°C for 35 days, respectively. After incubation, the broth with paired strains was stirred using a sterile 100 µl pipetter tip and plated onto M5 agar for differential enumeration. Plates were incubated anaerobically at 25°C for 36 hours. The colonies were counted separately by color (white or blue). Tests were repeated twice.

3.2.6. Statistical Analysis

Microbial data were analyzed using SAS analytical software (SAS Institute Inc., Cary, NC). The general linear models procedure (PROC GLM) was used for the analysis of variance and the differences between means were compared using Duncan's test.

3.2.7. Differential Enumeration of Bacterial Strains by PFGE

Strains No.3, No.10 and No.13 were grown in MRS broth at 25°C for 48 hours. Then 3 cultures were mixed together. Serial dilutions of the mixed broth were plated onto M5 agar. Plates with the appropriate number of colonies (100-300) were counted and further incubated anaerobically at 25°C for 48 hours to obtain colonies about 0.5-1.0 mm in diameter.

A convenient number of colonies (10-25) was randomly selected using Harrison's disc (Harrison, 1938; Figure 7). Each selected whole colony was picked up using a sterile platinum loop and put in 100 µl of 42°C low melting point agarose gel (1%) in a 1.5 ml Eppendorf tube. A cell suspension was made by vigorous vortexing using a Fisher Vortex Genie 2 vortexer. An agarose insert was made for each colony. One slice (0.5mm in thickness) of the insert for each colony was made. Two slices of blank gel were also made as control. All the slices were put in one sterile Eppendorf tube.

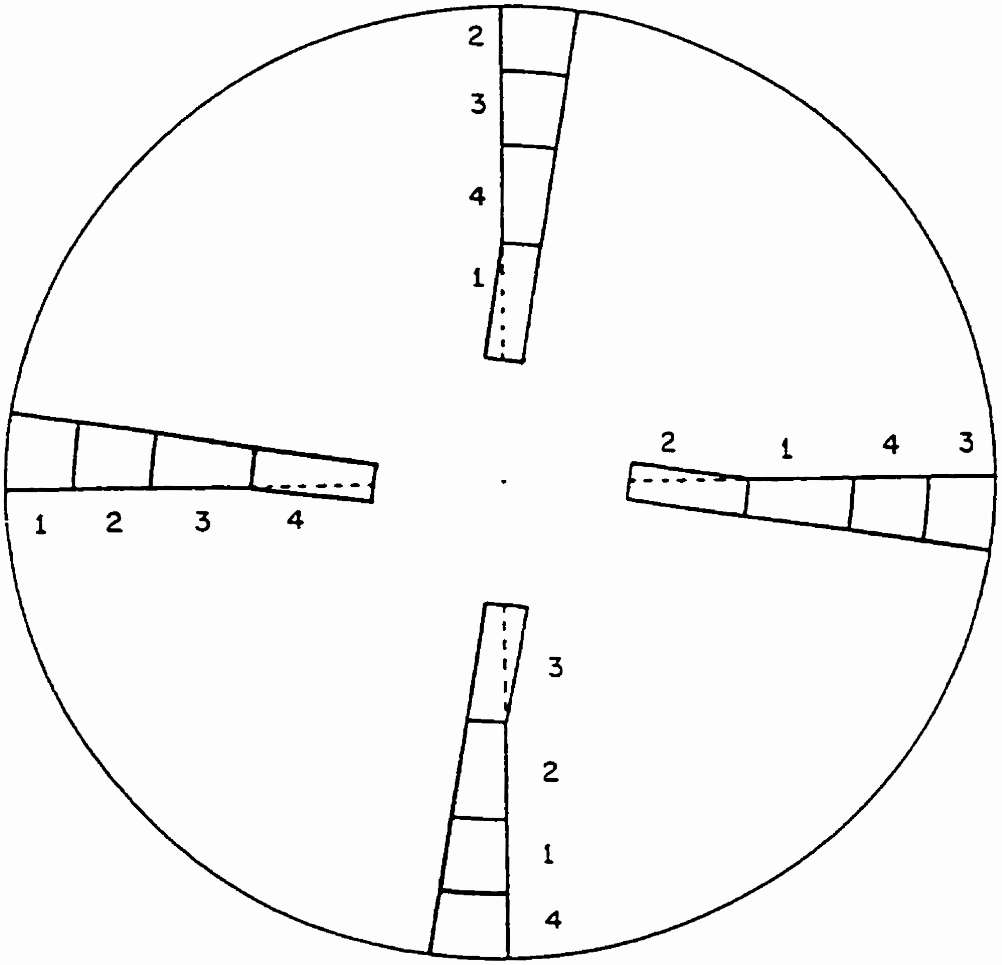


Figure 7. Harrison's disc showing the sampling area only. The areas of 1, 2, 3 and 4 are equal in size. The disc can be used as a template and the Petri-dish to be examined was placed centrally on the disc. Colonies were chosen in area 1, 2, 3 and 4 until a convenient number was gathered. All colonies lying over lines were ignored.

The DNA purification and digestion were performed as described previously. After digestion with *Sma*I, all the slices were loaded randomly into the running gel slots and the PFGE was performed as described previously.

The number of lanes that possessed the same band patterns was recorded and the band patterns were compared with known strain profiles to show the identity of strains under study. After acquisition of the ratios among these strains by PFGE, the approximate numbers in the whole plate were calculated and using these data, the quantity of each strain in the sample mixture was determined.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Strain Isolation

4.1.1. Isolation of LAB From Sliced Ham

Bacterial numbers in spoiled sliced ham (Smoked Pepper Loaf) samples are listed in Table 1. Three different packages (designated SPL1, SPL2 and SPL3) with the same expiry date were analyzed separately. There was little exudate present inside the package film and the color and texture of the sliced meat appeared normal. However, SPL2 exhibited a severely blown-package. The pH values of these products had decreased substantially from normal to 4.6-4.7. A sour odor could be easily perceived when the packages were opened.

Bacterial loads of all samples reached 8 logCFU/cm^2. Bacterial numbers on MRS agar were slightly higher than on APT agar with all the 3 samples. This was an indication that the microflora on the spoiled meat was primarily dominated by LAB- because MRS medium recovers only LAB (de Man, et al., 1960) but APT medium can be used to cultivate a variety of microorganisms including LAB. Among APT, MRS, MRSD and M5 media, MRS agar recovered the greatest number of LAB, with APT, MRSD and M5 agars containing 0.1-0.2 logCFU/cm^2 lower numbers than MRS. This is perhaps because the composition of MRS medium most favors the growth of LAB. However, data in Table 1 reported that all 4 media were suitable for the recovery of LAB. After 48 h anaerobic incubation at 25°C and post-staining with bromocresol purple, blue colonies on MRSD,

Table 1. Microbial analysis of vacuum-packaged sliced ham samples (Smoked Pepper Loaf, 30 days after expiry date).

Type of agar	Differential count	Ham sample (logCFU/cm^2)		
		SPL1	SPL2	SPL3
APT[1]		8.53	8.39	8.30
MRS		8.72	8.55	8.34
M5	total	8.68	8.25	8.23
	B[2]	8.66	8.12	8.20
	W[3]	7.20	7.64	7.05
	percent W	3.3%	24.6%	6%
MRSD[4] 25°C	B	<5[5]	<5	<5
	G	8.52	8.33	8.21
pH		4.63	4.71	4.66

[1] Dilutions plated on APT agar were incubated aerobically at 25°C for 48 h.

[2] Blue colonies (B) on M5 agar plates (incubated at 25°C anaerobically for 48 h) were homofermentative LAB.

[3] White colonies (W) on M5 agar plates were heterofermentative LAB.

[4] MRSD was used to differentiate *Pediococcus* from *Lactobacillus* or *Leuconostoc* spp. Blue colonies (B) would have been pediococci, green colonies (G) were *Lactobacillus* or *Leuconostoc* spp.

[5] Limit of sensitivity of the method.

which would have been pediococci, were not found.

Both homofermentative and heterofermentative LAB were found on M5 plates. Differential enumeration of these plates revealed that the number of homofermentative LAB was slightly higher than that of heterofermentative LAB. In sample SPL1 and SPL3, homofermentative LAB reached 8 log CFU/cm^2 while heterofermenters reached 7 log CFU/cm^2. The percentage of heterofermentative LAB was about 3-7%. However in SPL2, the percentage of heterofermentative LAB reached 24.6% which meant both groups shared dominance at spoilage. The growth of heterofermenters may have been the reason for its blown package and why the pH of SPL2 was slightly higher than the other two packages (Table 1).

Based on the color and size of the colonies, two different types of strains from each sample were isolated, subcultured and preserved at -80°C for later use. The size of white colonies (heterofermentative LAB) on M5 agar was slightly bigger than the blue colonies. The appearance (size and color) of these colonies was also consistent on other media. On MRS or APT plates, the heterofermentative strain formed big white colonies while the homofermentative strain formed small and yellowish colored colonies. On MRSD plates, although the color of the colonies were all green, the heterofermentative strain formed bigger colonies. The two different isolates were checked under the phase contrast microscope and heterofermentivity of the presumed heterofermentative isolate was confirmed by CO_2 production tests. The homofermentative organisms which formed blue colonies on M5 agar were short curved rods and the heterofermentative organisms that formed white colonies were cocci in pairs or chains. No spores were observed.

4.1.2. Isolation of LAB From Corned Beef

Two pieces of vacuum-packaged corned beef samples (about 2 kg per piece) were obtained from an Ontario-based national meats manufacturer. One piece of meat was manufactured with a standard formulation and the other one had extra sucrose added in an attempt to extend shelf life by changing the dominance of bacteria in the products. Both packages showed copious slime formation, but neither package was observed to be blown. About 50 ml of ropy slime and a surface sample of meat were collected from each package and analyzed separately. The bacterial numbers present are listed in Table 2. Results indicated that the pH values of the slime were slightly lower than the respective meat sample in each package, but the pH values of the slime or meat in both the sucrose-added sample and the control sample were similar. Thus the addition of sucrose did not affect the final pH of the meat at spoilage. There was also no substantial difference between the ropiness of the slime isolated from the two samples when the slime was touched with a loop to measure the maximum length of the rope (ca. 5 cm).

Just as in the sliced ham samples, comparable bacterial numbers on both APT and M5 agar indicated that LAB dominated the spoilage microflora of the corned beef. LAB levels in the slime in both samples reached 9 log CFU/mL, which was 1-2 log higher than that in the meat. It should be noted that the total numbers of bacteria present in the slime from the control sample (without additional sucrose) recovered on APT agar were one order ($\log_{10}$) lower than those in M5 plates. This did not happen in the sucrose-added sample. Maybe in this case APT medium (aerobic) is not suitable for the recovery of these spoilage organisms. The absence of blue colonies on MRSD agar indicated that pediococci

Table 2. Microbial analysis of commercially manufactured, vacuum-packaged corned beef samples.

Medium		sucrose-added sample[1]		control sample[2]	
		slime (logCFU/ml)	meat (logCFU/cm^2)	slime (logCFU/ml)	meat (logCFU/cm^2)
APT[3]		9.30	7.90	8.58	7.62
MRSD (blue)[4]		<5	<5	<5	<5
M5	total	9.40	7.97	9.41	7.76
	B[5]	9.27	7.81	9.24	7.64
	W[6]	8.81	7.45	8.91	7.15
	percent W	25.5%	30.2%	31.6%	24.5%
Thermo-tolerant aerobes[7]		5.73	3.72	5.11	3.85
Thermo-tolerant anaerobes		5.52	3.67	5.52	3.36
pH		5.14	5.26	5.09	5.24

[1] 25 days after expiry date.

[2] 29 days after expiry date.

[3] Dilutions inoculated on APT agar were incubated aerobically at 25°C for 48 h.

[4] MRSD was used to differentiate *Pediococcus* from *Lactobacillus* and *Leuconostoc* spp. Blue colonies would have been pediococci.

[5] Blue colonies (B) on M5 agar plates (incubated at 25°C anaerobically for 48 h) were homofermentative LAB.

[6] White colonies (W) on M5 agar plates were heterofermentative LAB.

[7] Dilutions were placed in a 75°C water bath for 15 min, spread-plated on APT and incubated at 25°C for 48 h both aerobically and anaerobically.

did not dominate in the final spoilage of these products. Since they are not usually able to grow at temperatures below 8°C, sustained temperature abuse was not likely a factor influencing spoilage.

Both homofermentative and heterofermentative LAB established high levels of growth in these packages in both the slime and on the meat with the number of homofermentative LAB being higher (no more than one order). This dominance pattern is similar to what was observed in the sliced ham samples. However, in this investigation, the percentage of heterofermentative LAB reached about 25-30% of the total bacteria present.

A comparison of the pH values and the LAB numbers between the sliced ham samples and corned beef samples revealed that at a similar level of viable bacterial numbers, the pH drop was very different in these two spoilage cases. The pH values of the corned beef stayed above 5 while the pH of the sliced ham dropped to 4.6 - 4.7. This is probably because the sliced ham had spoiled to such an extent that the bacterial load began to decrease in response to the great amount of acid produced. That is to say, the total LAB level may have been much higher at some point before the sampling took place. In a study relating spoilage and pH values of vacuum-packaged cooked ring sausages (Korkeala et al., 1990), the initial pH of the meat was about 6.3. When the meat was deemed to be spoiled, the pH had dropped to 5.8 -5.9 and the lowest pH value observed was 4.6. When the pH dropped below 5.0, the LAB level was between 8-10 log CFU/g. This is quite similar with the results obtained in present study. However, it should be noted that pH can not be used to reliably define spoilage in these meat products (Kempton and Bobier, 1970).

Thermo-tolerant aerobes and anaerobes were also recovered on APT agar, following heat treatment of the sample dilutions at 75°C for 15 min. Similar numbers of bacteria and colony size and color on plates incubated with different atmospheric conditions indicated that these thermo-tolerant bacteria were LAB. Compared with the previous total numbers on M5 agar, a 4 log reduction was achieved on both corned beef samples after the heat treatment. Therefore it is unlikely that thermo-tolerant organisms were a major factor in the development of product spoilage.

Based on colony appearance, a total of 14 isolates were collected from the M5 plates (Table 3). Five different types of isolates (3 homofermentative and 2 heterofermentative bacteria) were found on M5 plates from each sample. However, only 2 types of isolates (one homofermentative and one heterofermentative LAB) were recovered from the samples following heat treatment. It should be noted that the color of the colonies on M5 agar sometimes was not very stable and consistent, and appeared to be strain-dependent. Strict anaerobic conditions were required and different strains needed different incubation times to achieve full color development (Zuniga et al., 1993). Therefore, sometimes the homofermentative strains appeared as white colonies with blue centers of various sizes. Cell morphology was found to be very helpful for confirming colony type in conjunction with the color information - although it was quite tedious.

It was also noticed that the ropy slime-forming isolates from both the sucrose-added and the control samples had lost their ropy slime producing ability when grown on all types of media used in this study. In the investigation of the ropy slime-producing *L. sake* isolated from Finnish ring sausages, the ability of *L. sake* bacterial colonies to form slimy ropes when touched with a loop under certain incubation conditions, has been used as one

Table 3. LAB strains isolated from commercial corned beef samples from M5 plates.

Colony appearance	Designation of isolates	
	Sucrose-added sample	control sample
White colony[1]	CB1	CB1'
White colony[2]	CB2	CB2'
Dark blue colony	CB3	CB3'
Light blue colony	CB4	CB4'
Blue colony[2]	CB5	CB5'
White colony (thermotolerant)	CB6	CB6'
Blue colony (thermotolerant)	CB7	CB7'

[1] Colony with a diameter of 1.0-1.5 mm.

[2] Colony with a diameter of 0.3-0.6 mm.

criterion to establish a semi-quantitive method for assessing slime production capability (Makela and Korkeala, 1992). Wide variation has been observed in the appearance of ropiness on different agars with different incubation conditions. Some of the strains capable of forming slime in vacuum packages did not form ropy colonies under any of the test conditions (Makela and Korkeala, 1992), which was the same situation as observed in this study. It was noted that the only reliable way of testing the slime producing capabilities of different strains was an incubation test using vacuum-packaged sterile meats (Bjorkroth et al., 1996).

4.2. Stain Identification

4.2.1 Biochemical Identification of Isolates

Morphological characteristics and key biochemical reactions that permitted identification of isolated strains from the spoiled sliced ham and corned beef samples are reported in Table 4. All the strains were Gram positive and catalase negative, which are major characteristics of LAB. The three cocci isolated from sliced ham and 6 cocci from corned beef samples were unable to hydrolyze arginine but able to produce gas from glucose. These organisms were identified as *Leuconostoc* spp. according to the identification scheme developed by Schillinger and Lucke (1987, Figure 2). Other microorganisms isolated from the spoiled meat were all rods and were not able to produce CO_2 from glucose. This latter group belonged to the homofermentative lactobacilli according to that identification scheme. The rods isolated from spoiled ham were narrower and longer than those isolated from corned beef and were therefore different. None of the

Table 4. Source, morphological and biochemical characteristics of LAB strains isolated from spoiled meats.

Source	Sliced ham						Corned beef														
	SPL1		SPL2		SPL3		Sucrose-added							Control							
Isolates	R1	C1	R2	C2	R3	C3	CB1	CB2	CB3	CB4	CB5	CB6	CB7	CB1'	CB2'	CB3'	CB4'	CB5'	CB6'	CB7'
Cell shape	r[1]	c[2]	r	c	r	c	c	c	r	r	r	c	r	c	c	r	r	r	c	r
Spores	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gram test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CO_2[3]	-	+	-	+	-	+	+	+	-	-	-	+	-	+	+	-	-	-	+	-
Arginine	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	+	+	+	-	+
Growth at 8°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 30°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 45°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[1] r: rods; [2] c: cocci; [3] CO_2: CO_2 production from glucose.

homofermentative lactobacilli isolated from the spoiled ham were able to hydrolyze arginine but all the rods from the corned beef could.

Because it was presumed that the 3 rods and 3 cocci isolated from the 3 different packages of sliced ham were just two different organisms and the corresponding isolates from the corned beef control sample were the same as the isolates from the sucrose-added sample, only isolates R1, C1, CB1-CB7 were chosen for the API 50 CHL test. The API test results are listed in Appendix 5.

Except for strains CB6 and CB7, which shared the same sugar fermentation profile with CB2 and CB3, respectively, other organisms showed distinctive sugar fermentation patterns in the API tests which indicated that they were different strains. Comparison of sugar fermentation profiles with the commercially provided identification key (bioMérieux S.A., Marcy l'Etoile, France) revealed that the 4 *Leuconostoc* strains (C1, CB1, CB2 and CB6) belonged to *Leuconostoc mesenteroides* ssp. *mesenteroides* and strain R1 was a *L. curvatus* strain. It should be noted that there was no identification profile available for *L. sake* in the data provided with the API 50 CHL test kits. However, the fermentation profiles of isolates CB3, CB4, CB5 and CB7 closely resembled the published profile of *L. curvatus* except that all of these isolates were melibiose, sucrose and trehalose positive, which are the key differences between these two species. *L. sake* strains usually metabolize these latter sugars. (Kandler and Weiss, 1986). These 4 isolates were also arginine positive (Table 4). This is another key criterion to differentiate *L. sake* from *L. curvatus*. Both Kandler and Weiss (1986) and Hugas et al. (1993) found that 90% of *L. sake* strains from their collections showed positive results in arginine hydrolysis tests while 90% of the *L.*

curvatus were arginine negative. Based on the API sugar fermentation profiles and the results of arginine tests, isolates CB3, CB4, CB5 and CB7 were clearly identified as *L. sake*. It was also observed that all of the 4 *L. sake* strains were gluconate positive. This property was also documented by Schillinger and Lucke (1987) who found that 98% of *L. sake* were gluconate positive while only 37% *L. curvatus* yielded positive results. Strain R1 which was identified as *L. curvatus* in this study was not able to ferment gluconate. The 4 *L. sake* isolates all produced CO_2 from gluconate but not glucose, and this is consistent with Stiles and Holzapfel's (1997) statement about the heterofermentivity of *L. sake*.

Since the paired strains CB2 and CB6 plus CB3 and CB7, respectively, shared identical API 50 CHL profiles and since CB6 and CB7 were selected from the original spoilage population (Table 3), it was suspected that CB6 and CB2 were the same *Leuconostoc* strain and CB7 and CB3 were the same *L. sake* strain. However, this hypothesis needed to be confirmed by PFGE analysis. In addition, the PFGE technique was planned to be used to test the hypothesis that the rods and cocci isolated from the 3 different packages of sliced ham were the same respective strain and that the corresponding isolates from the corned beef control sample were the same as the isolates from the sucrose-added sample. Since PFGE analysis can be one of the more informative methods for bacterial strain characterization (Farber, 1996; Tenover et al., 1995) it was a logical choice as a method to use for further study of these isolates.

4.2.2. PFGE Identification of Spoilage LAB Isolated From Vacuum-Packaged Cured Meat Products.

4.2.2.1. Comparison and interpretation of the PFGE results

A number of spoilage LAB isolates including the LAB strains isolated from previous microbial analysis of sliced ham and corned beef, as well as strains isolated from retail cured meats in Quebec (Holley et al., 1995), plus standardized cultures obtained from Kulmbach, Germany (No.14, 15) and ATCC type strains (No.33, 34 and 35) were included in this study. Genomic DNA of these isolates was purified, digested with the restriction endonuclease *Sma*I and electrophoresed in a Gene Navigator PFGE system (Pharmacia Biotech AB, Uppsala, Sweden). Some of the gel pictures are presented in Figures 8, 9 and 10.

A consolidation of PFGE results of the LAB isolates from spoiled sliced ham and corned beef is presented in Table 5. The results of the PFGE analysis showed that the band patterns of R1, R2, and R3 were identical. A similar result was obtained within the group C1, C2 and C3 (data not shown). This verified the hypothesis that R1, R2 and R3 were the same strain and that the three coccoid isolates C1, C2 and C3 were also one strain. Therefore, R1, R2 and R3 were finally designated as strain R, and C1, C2 and C3 were designated as strain C.

The PFGE analysis also confirmed that the corresponding isolates (initially based on the appearance of the colonies) in the corned beef control sample were identical with the strains isolated from the sucrose-added sample (Figure 11). Paired strains CB2 and CB6 as well as CB3 and CB7, which had identical API sugar fermentation profiles also appeared identical in PFGE analysis. Strains that were different from each other in the API analysis were also different from each other in PFGE analysis. As a result, strains CB1 and CB1'

Figure 8. PFGE patterns of the genomic DNA digested with *Sma*I for strain No.3, 4, 5, 10, 13. The central lane marked with "M" is the size standard. The unit of the numbers is kb.

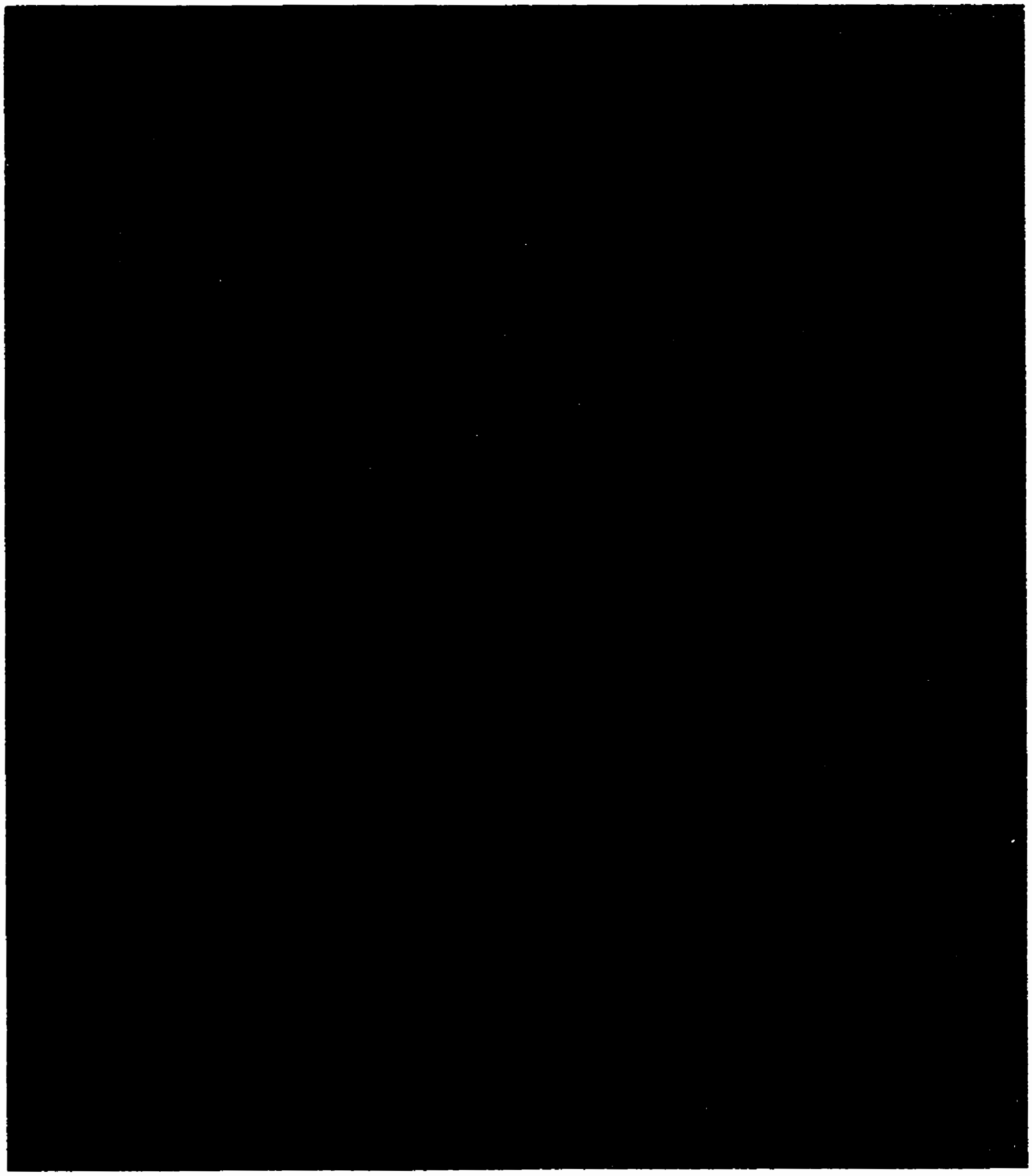


Figure 9. PFGE patterns of the genomic DNA digested with *Sma*I for strains No.3, 4, 5, 10, R, 23, C, 13, 11, 1 and 6. The central lane marked with "M" is the size standard (kb). 3N and 4N are PFGE patterns of strains No.3 and No.4 from new frozen stock. The DNA sample of No.11 was contaminated and partially degraded prior to electrophoresis.

Figure 10. PFGE patterns of the genomic DNA digested with *Sma*I for strains No.33, 11, 35, 15, 5, 6, 9, 14 and 34. The central lane marked with "M" is the size standard (kb). *Leuconostoc mesenteroides* ssp. *mesenteroides* strain No.33, *L. sake* strain No.34 and *L. curvatus* strain No.35 are ATCC type cultures.

Table 5. Re-established bacterial strain groups obtained by PFGE of *Sma*I DNA digests from spoilage LAB.

New code for LAB strains	Strains sharing the same biochemical profile	Strains sharing the same PFGE profile
R	R1, R2, R3	R1, R2, R3
C	C1, C2, C3	C1, C2, C3
CBLc1	CB1, CB1'	CB1, CB1'
CBLc2	CB2, CB2' CB6, CB6'	CB2, CB2' CB6, CB6'
CBLb1	CB3, CB3', CB7, CB7'	CB3, CB3', CB7, CB7'
CBLb2	CB4, CB4'	CB4, CB4'
CBLb3	CB5, CB5'	CB5, CB5'

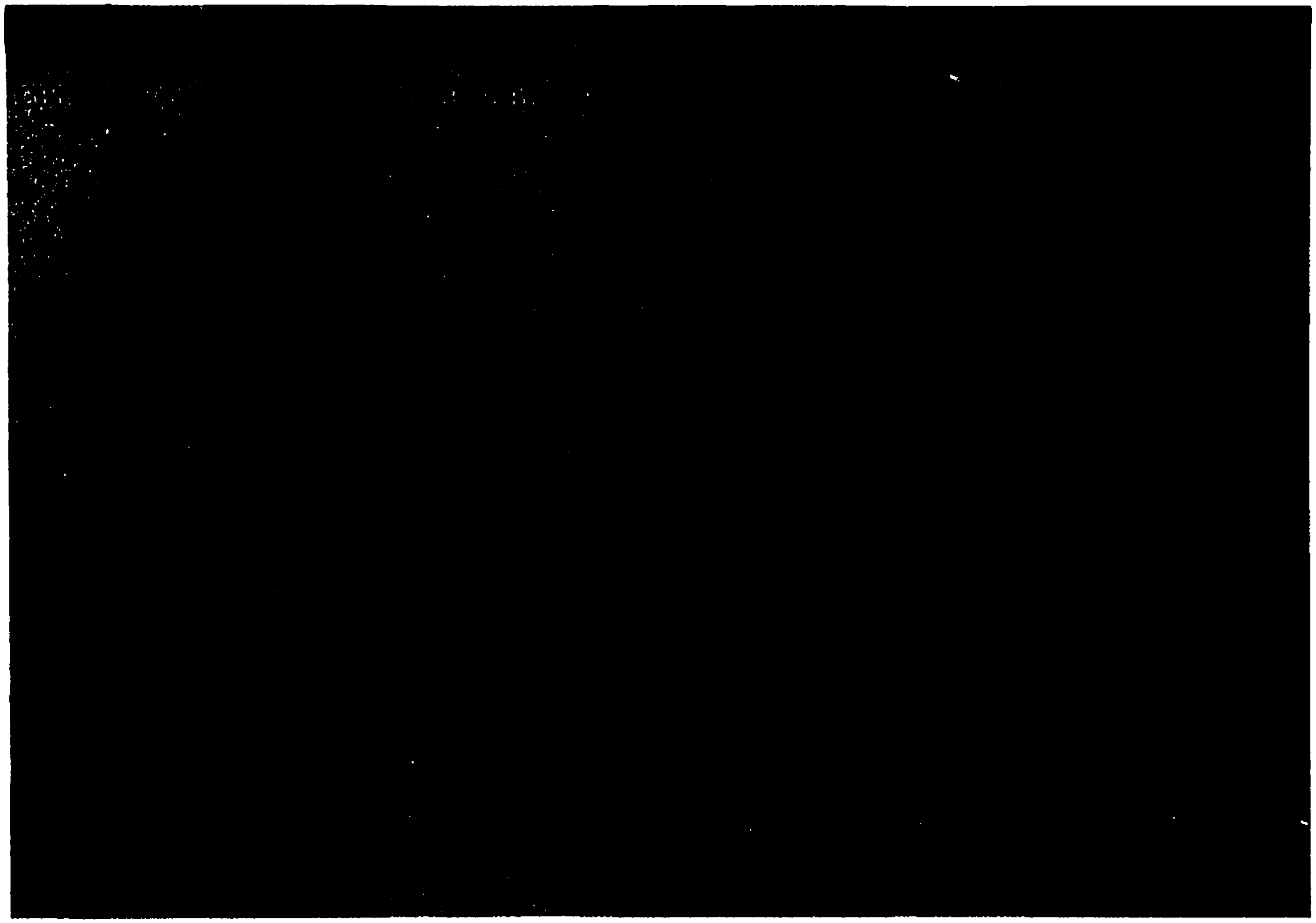


Figure 11. Comparison of PFGE profiles of LAB strains isolated from corned beef samples. This is a composite gel image edited using 3 separate PFGE results. The designation of the isolates is listed in Table 3. It is shown that CB6, CB6', CB2 and CB2' share the same PFGE gel pattern. CB7, CB3, CB3' and CB7' share the same PFGE pattern. CB1 and CB1', CB5 and CB5', CB4 and CB4', respectively, are also validated as the same strains. M is the size standard (kb).

were designated strain CBLc1. Strains CB2, CB6, CB2' and CB6' were all designated CBLc2. Strains CB3, CB7, CB3' and CB7' were all designated CBLb1. Strains CB4 and CB4' were renamed CBLb2. Strains CB5 and CB5' were designated CBLb3. It was noticed that among the 3 *Lactobacillus* and 2 *Leuconostoc* strains isolated from the corned beef samples and characterized by API and PFGE, strain CBLb1 and CBLc2 were thermo-tolerant LAB.

In another investigation of ropy slime producing *L. sake* strains, 67 strains which showed identical API profiles were identified as 7 different groups based on PFGE analysis using two different enzymes (Bjorkroth et al., 1996); but no two strains from different phenotype groups showed an identical PFGE profile. This indicates that compared with the API test, PFGE has higher discriminatory power and can provide more information for strain differentiation.

It was also noticed that strains No.3 and 4, previously identified as *L. curvatus* and *L. sake*, respectively (Holley et al., 1995), showed an identical PFGE pattern with *Sma*I digestion. These two strains had similar morphology and growth characteristics. Biochemical tests specially keyed for differentiation of *L. curvatus* and *L. sake* showed identical results which indicated that both strains belonged to the species *L. curvatus* (Table 6). Frozen replicates of original culture collection material of these two strains were cultured and subjected to a second PFGE analysis using both *Sma*I and *Avr*II separately as the rare genome cutter. The results still showed identical profiles following both *Sma*I and *Avr*II digestion (Figure 12). Finally, the conclusion that strain No. 3 and No. 4 were the same strain (*L. curvatus*) was made because the probability is very low that different LAB

Table 6. Biochemical tests for differentiation of strain No. 3 and No.4.[1]

Strain	No.3	No.4	Reference strain			
			No.14	No.15	No.34	No.35
Arginine	-	-	+	-	+	-
Arabinose	-	-	+	ND	ND	ND
Melibiose	-	-	+	ND	ND	ND
Trehalose	+	+	+	ND	ND	ND
Amygdalin	+	+	+	ND	ND	ND
Maltose	+	+	+	ND	ND	ND

[1] No.14 and No. 34 are known *L. sake* cultures. No.15 and No.35 are known *L. curvatus* cultures. "ND": not determined.

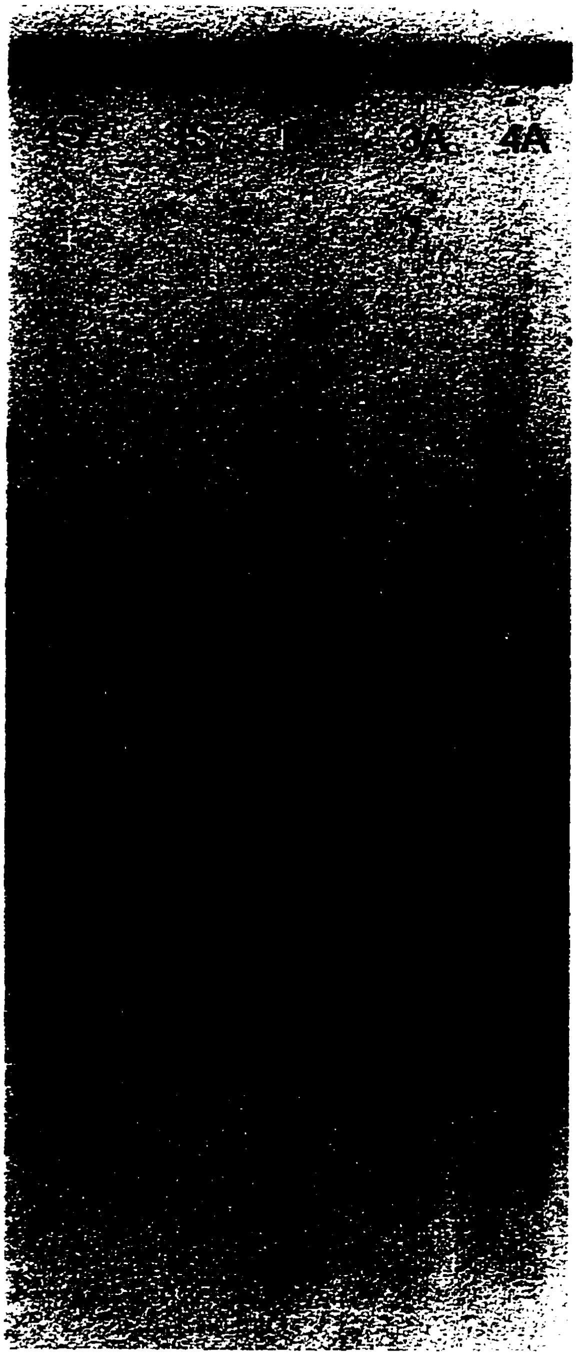


Figure 12. PFGE patterns of the genomic DNA digested with *Sma*I and *Avr*II for strains No.3 and No.4. Lanes 3S and 4S are strains No.3 and No.4 digested with *Sma*I. Lanes 3A and 4A are strains No.3 and No.4 digested with *Avr*II. Lane "M" is the size standard (kb). *Avr*II generated a wider range of DNA fragments but within the digestion by the same enzyme, strains No.3 and No.4 share identical profile.

strains will show the same PFGE profile after digestion with two different enzymes. From this analysis, it was noted that the reproducibility of PFGE was excellent. Given the same enzyme digestion and electrophoresis conditions, consistent band patterns were achieved in different runs. Therefore, the developed strain profiles are valuable and can be used as a reference for later analyses or to build a PFGE profile library.

Unlike the *L. sake* strain groups analyzed by Bjokroth et al. (1996), which showed only one or two band differences in their PFGE profile, the different strains in the present study possessed a diversity of band patterns. Namely, all the strains within or among species could be differentiated easily by visual comparison. The percentage similarity of band patterns was less than 25% (data not shown). This is an indication that these strains are not genetically closely related to each other.

By visual comparison, it was very easy to tell the difference between any two strains and the overall difference between *Leuconostoc* and *Lactobacillus* spp. was obvious. However, differentiation of *L. sake* from *L. curvatus* as a whole based on the gel patterns is problematic. The sizes of the bands digested with *Sma*I for both *L. curvatus* and *L. sake* were in the same range (from ca.130 kb to 6 kb), with the average size range of *L. sake* being a little bit smaller (from ca.110 kb to 6 kb). However, *L. curvatus* strains such as No. 10 also had bands in a small size range (ca.90 kb to 6 kb). No unique band or combined band patterns were found in either species that enabled effective species identification by itself. The differentiation of single strains was based on the band size (location) differences within the band patterns. The differentiation of *Leuconostoc* spp. from *Lactobacillus* spp. was based on the overall size ranges (The size of *Leuconostoc* DNA digests ranged from ca.

230 kb to 10 kb). This difference was generated by the significant genome size and sequence differences of these two genera. There is low DNA-DNA homology between these two genera. The mol% G+C in the genomic DNA is 37-39 for *Lc. mesenteroides* (Garvie, 1986) but is 42-44 for both *L. curvatus* and *L. sake* (Kandler and Weiss, 1986). Although *L. curvatus* and *L. sake* were recognized as two well defined species by DNA-DNA homology (Klein et al, 1996), they do not possess such significant genomic heterogeneity that they could show band pattern differences as if they were two different genera, especially when DNA-DNA hybridization has shown that these two species share a very high level of homology (Kandler and Weiss, 1986). However, the conclusion from the DNA-DNA homology study is more informative than just the macrocleavage patterns of the genomic DNA at the species level and is complementary.

A dendrogram showing the genetic distance between 7 *Lc. mesenteroides* strain types by PFGE was constructed using software provided by Dr. Brian Fristensky from the Plant Science Department, University of Manitoba. First, the image data of the band patterns of these strains were converted to binary information (0, 1) in a spread sheet which can be recognized by this software (Table 7). Using the restriction sites maximum likelihood (RESTML) method (Felsenstein, 1992), a dendrogram was constructed for these 7 strains (Figure 13). In this phylogenetic tree, the branch length among strains defines the genetic distance of these strains (Felsenstein, 1992). It is noted that strain No.1 and No.11, which were both isolated in Quebec and able to produce bacteriocin, shared a relatively close relationship. However, strain CBLc1 and CBLc2 which were both isolated from the corned beef sample, belonged to different clusters in this analysis. In the PFGE analysis of ropy

Table 7. Binary information converted from PFGE gel profiles of 7 *Leuconostoc mesenteroides* strains which was used as the database to construct a dendrogram.[1]

No. of bands	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
Strain No.1	0	1	0	0	0	1	0	0	1	1	0	1	0	0	1	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0	1	0	0	0
Strain No.11	1	0	0	0	0	1	1	0	1	1	0	1	0	0	1	1	0	0	1	0	0	1	0	0	1	0	0	0	0	1	1	0	1	0	1	0	0	0
Strain No.13	0	1	0	1	0	0	1	0	1	1	0	0	1	0	1	1	1	0	0	1	0	0	0	1	1	1	1	1	1	1	1	0	0	1	1	1	1	0
Strain No.33	0	1	0	0	0	1	0	1	0	0	1	0	1	0	1	1	1	0	0	0	1	1	1	0	0	1	0	1	0	1	1	1	0	1	1	0	0	1
C	0	1	0	0	0	0	1	0	0	1	0	1	1	0	1	1	1	0	1	0	0	0	1	0	1	0	0	0	1	1	1	0	0	1	1	1	1	0
CBLc1	0	1	1	0	1	0	1	0	0	1	0	0	1	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1	0	1	1	0	1	0	0	0
CBLc2	0	1	0	1	0	0	1	0	0	0	0	1	0	1	1	1	1	0	1	0	0	0	0	0	1	0	0	0	1	1	1	0	1	0	1	1	1	0

[1] In the table "1" means the presence of band at a defined location while "0" means the absence of band at that location.

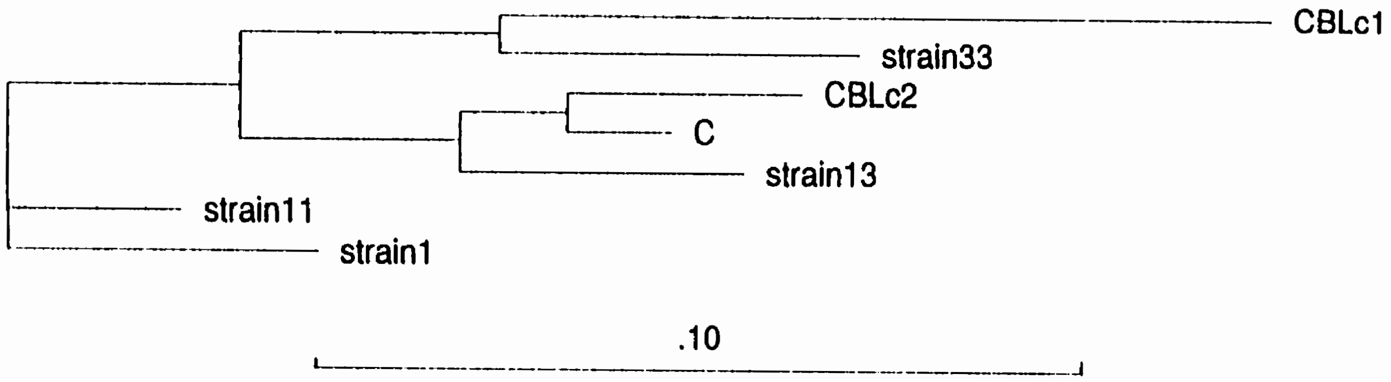


Figure 13. A dendrogram constructed using restriction sites maximum likelihood(RESTML) method (version 3.572c). The length of the branches on the tree shows the genetic distance among these *Leuconostoc* strains.

slime-producing strains isolated from Finnish ring sausages by Bjorkroth et al. (1996), all the strains were very closely related (having one or two band differences in the PFGE profiles). According to the interpretation by Tenover et al.(1995), these strains might have evolved from one single strain. For the 2 *Leuconostoc* strains isolated from the corned beef, there were at least 10 band differences between their PFGE patterns (Figure 11 and Table 7). Strain CBLc1 formed larger colonies on M5 agar while CBLc2 was more thermo-tolerant. There were also substantial differences in their API 50 CHL profiles. Among the 12 sugars that each of them utilized, these two strains had only 2 sugar fermentation test results in common (sucrose and D-turanose). This implies that a diversity of contamination sources might be present in the meat processing plant where these samples of meat were prepared.

4.2.2.2. Factors contributing to the quality of the PFGE analysis result

Several different factors can affect the quality of the PFGE analysis. Improper preparation of DNA samples will lead to smeared lanes and bad resolution. Usually 10^8 cell/ml is the proper concentration of a bacterial sample to achieve good results. An excessively high DNA concentration will waste restriction endonuclease which is expensive and cause incomplete digestion, yielding poor resolution. If the DNA concentration is too low, the signal in the gel image will be too weak to analyze. Intensive washing with different buffers used in the DNA purification and digestion steps is important to get very clean DNA blocks by removing all the protein and RNA, thus avoiding smeared lanes. A fresh stock of PMSF solution should always be made monthly to make sure the proteinase

K is completely inactivated before restriction enzyme digestion is initiated.

The proper loading of DNA slices into the wells is also very important to get accurate results. The slices should be loaded flat against the front wall in the well. If there are air bubbles in the well, it will very likely yield bad resolution. If the initial location of the slices in the wells is slightly different, the final band mobility of different samples can also be affected. For example, from Figure 8 it was observed that although strain No. 3 and No. 4 shared identical band patterns, (i.e., the band migration distances were identical in the two lanes), the corresponding bands were not in the same position in the lanes.

Sometimes when the DNA bands are not sharp enough it is better to use a 1.5% agarose running gel instead of 1.2% to obtain better resolution without significantly increasing the run time. Lower electrical current during electrophoresis is recommended to obtain better resolution. When the voltage and the concentration of the TBE buffer was fixed (200 V and 0.5xTBE), reducing the volume of the buffer from 2.5 l to 2.3 l was found to be an effective way to lower the current by increasing the overall electrical resistance of the electrophoresis system.

Proper programming of the pulse durations and run times is critical to obtain optimal separation. The relationship between pulse duration and resolution for the hexagonal electrode arrangement in the Gene Navigator system is listed in Table 8. By combining different pulse durations and run times, satisfactory resolution of a variety of size ranges of DNA digests can be achieved.

4.3. Bacteriocin Production and Resistance

Table 8. Relationship between pulse duration, run time and resolution for a hexagonal electrode. (Adopted from the Gene Navigator® User Manual, Pharmacia Biotech AB, Uppsala, Sweden)

Pulse duration	Efficient separation	Maximum resolution	Run time (h)	Set voltage (V)
0.3 s	1-10 kb	1-10 kb	1	450 (0.15xTBE)
0.5 s	5-30 kb	10-25 kb	2	450 (0.15xTBE)
0.8 s	30-50 kb	35-50 kb	3	450 (0.15xTBE)
5 s	20-100 kb	60-90 kb	4	300
25 s	40-400 kb	200-300 kb	6	300
45 s	40-600 kb	400-550 kb	10-24	300
100 s	40-1000 kb	700-900 kb	17-40	165-200
125 s	100-1600 kb	800-1200 kb	17-40	165-200
20 min	1-2.5 Mb	1.6-2.5 Mb	100-140	165-200
30 min	1.6-3 Mb	2.5-3 Mb	100-140	165-200
40 min	1.6-6 Mb	2.5-6 Mb	140	30
75 min	2-9 Mb	3-6 Mb	140-170	30
90 min	2-10 Mb	6-9 Mb	185	30
100 min	3-13 Mb	7-10 Mb	190-200	30 (0.6% agarose)
180 min	3-13 Mb	10-13 Mb	190-200	27 (0.6% agarose)

The buffer used was 0.5xTBE and agarose concentration was 1.2~1.5% (w/v) unless a different value is stated.

4.3.1. Agar Spot Test for Bacteriocin Screening

A total of 20 strains which were listed in the beginning of Chapter 3 were used as both test and indicator strains in this preliminary bacteriocin screening experiment. The results of interest are presented in Table 9. In this 20x20 screening test, only strain No. 1 and No.11 (which are two *Lc. mesenteroides* strains) as well as a *L. sake* strain CBLb2 (isolated from spoiled corned beef) showed inhibitory effects against some indicator strains. On the other hand, only *L. sake* strain No. 6 and No. 9 and *L. curvatus* strain No. 10 showed susceptibility to those 3 producer strains. Except in the antagonism tests where strain No.1 was used against No.6, and No.11 against No.6 where the width of the cleared zone was within 0.5-1.0 mm, the inhibition zones in all other tests was quite substantial (zone width > 5.0 mm, Figure 14).

Although the test medium was buffered with 1% morpholinoethane sulphonic acid (MES) to minimize the antibacterial influence of organic acid production, other inhibitory factors such as H_2O_2 can give confusing positive results in this test method. However, the agar spot method is fast, convenient and obvious, so it is suitable for preliminary bacteriocin screening for a large number of bacterial strains. After this screening, the positive strains can then be tested by other methods to validate bacteriocin production.

4.3.2. Well Diffusion Test

In this experiment, no inhibition zone was found, even in untreated culture supernatants. The culture supernatant (MRS) was then concentrated 10-fold by freeze-drying but there was still no positive reaction. It seemed that these 3 inhibitory substance-

Table 9. Inhibition of indicator strains by bacteriocin-producer strains in the agar spot test[1].

Producer strain	Inhibition of indicator strains[2]		
	No.6	No.9	No.10
No.1	(+)	+	+
No.11	(+)	+	+
CBLb2	+	+	+

[1] The conditions used for the agar spot test are described in Chapter 3.

[2] Symbols: "+" large inhibition zone (>2.0 mm); "(+)" small inhibition zone (ca. 0.5 mm). Strains: No.1 and No.11 were *Lc. mesenteroides*. No.10 was *L. curvatus*. No.6, No.9 and CBLb2 were *L. sake*.

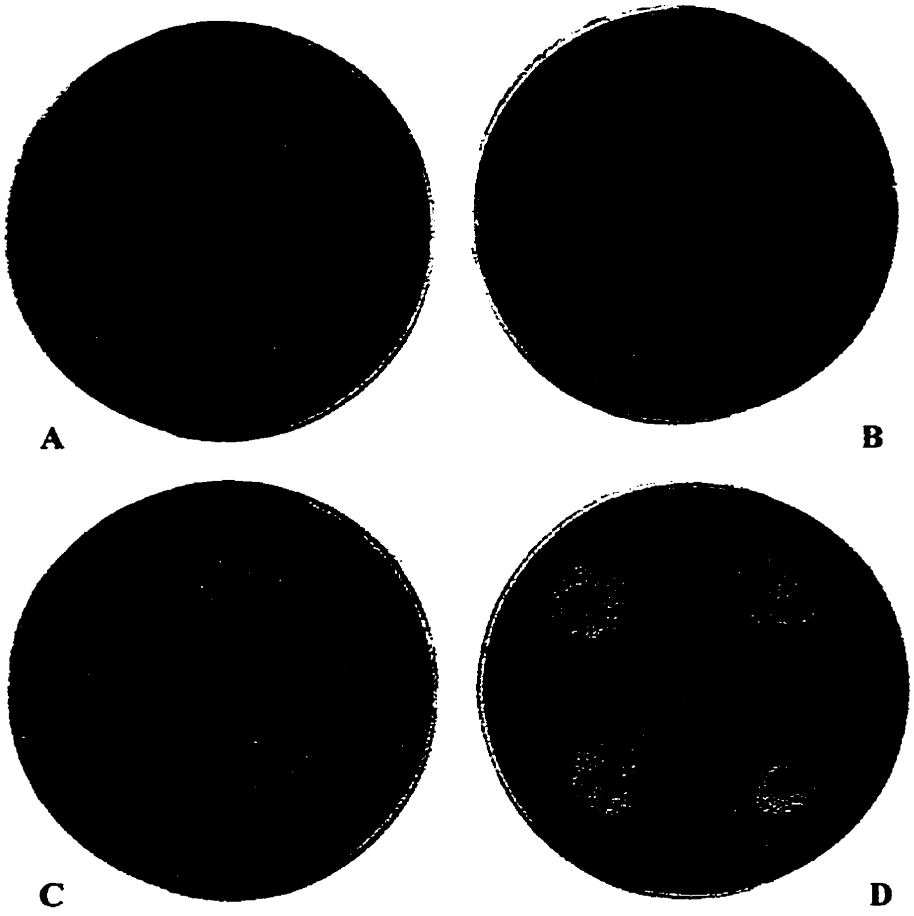


Figure 14. Agar spot test for the screening of bacteriocin production and resistance. Scanned picture shows plates containing inhibitory zones. In plates A, B and D, strain No.6 was used as the indicator strain and No.11, 1, and CBLb2 were used as the producer strains, respectively. In plate C, strain No.9 and No.11 were used as the indicator and producer strains, respectively. Except plate A and B on which the width of the clear zones were within 0.5-1.0 mm, the inhibitory effect of the other tests was very substantial, such as the test results in plate C and D (zone width >5.0 mm).

producing strains (No.1 and No.11 and CBLb2) did not secrete the antibacterial compound into the liquid medium under test conditions or secreted such a small amount that it could not be detected by the well diffusion test. This was not an unusual result. In a study of antibacterial activity of *L. sake* strains from meat, a total of 19 out of 142 *L. sake* strains exhibited antagonistic effects on solid agar medium, but only 6 strains were shown to secrete the inhibitory compound when grown in MRS broth (Schillinger and Lucke, 1989). Similar results were also obtained by Geis et al. (1983), who tested 93 strains of LAB and found that 36 strains exhibited antagonistic effects on agar, but only one strain produced an inhibitory substance in liquid medium.

4.3.3. Modified Agar Spot Test for Confirmation of Bacteriocin Production

In this experiment, catalase and 3 different proteases were used separately to eliminate any inhibitory activity resulting from the production either H_2O_2 or bacteriocins. Catalase or proteases were placed closely beside a producer strain colony grown on the agar surface. If the reagent inactivated the previously diffused antibacterial compound around the colony, the indicator strains were able to grow at the site of its inactivation. Therefore, an inhibition zone with asymmetric outline could be observed. The test results of this experiment are listed in Table 10, and the scanned image of some representative plates is shown in Figure 15.

Catalase was unable to neutralize the antibacterial activity of producer strains tested. This ruled out the possibility that antibacterial activity was due to the presence of H_2O_2. All the 3 proteases tested (proteinase K, protease and trypsin) gave an asymmetric inhibitory

Table 10. Inactivation of antibacterial substances produced by meat spoilage LAB strains by a modified agar spot test[1].

	Inactivation of antibacterial compound[2]					
Producer strain	No.1		No.11		CBLb2	
Indicator strain	No.9	No.10	No.9	No.10	No.9	No.10
Control	-	-	-	-	-	-
Catalase	-	-	-	-	-	-
Proteinase K	+	+	+	+	+	+
Protease	+	+	+	+	+	+
Trypsin	+	+	+	+	+	+

[1] The conditions used for this test are described in Chapter 3.

[2] Symbols: "+" formed asymmetric outline around the clear zone; "-" no asymmetric outline around the clear zone.

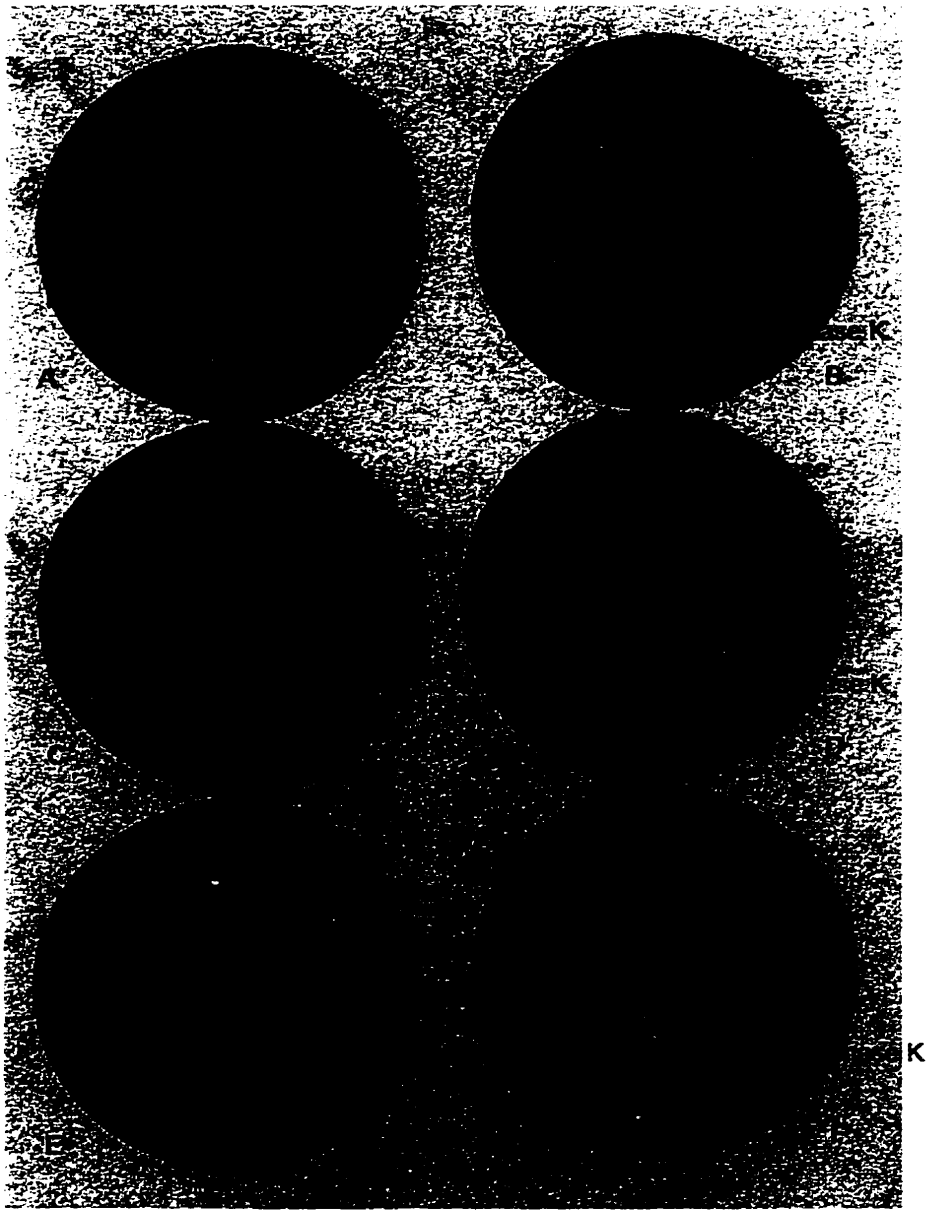


Figure 15. Modified agar spot test showing the effect of catalase, proteinase K, protease and trypsin on the inhibitory substance produced by LAB strains No.1 and No.11. Plates A and B show the inhibition zones produced by strain No.11 using No.9 as the indicator strain. Plates C and D show the inhibitory effect of No.1 using No.9 as the indicator strain. Plates E and F show the inhibition zones produced by No.1 on a lawn of No.10. A colony surrounded by an asymmetric clear zone was considered a positive result. A bacteriocin-positive strain should be catalase negative and protease sensitive. The procedures used in this test are described in Chapter 3.

zone outline in every test. These results proved that the antibacterial substances were proteinaceous. These results were also consistent with the literature (Yang and Ray, 1995; Kelly et al., 1995) where frequent isolations of spoilage LAB with bacteriocin activity have been made. Although the heat resistant property of these substances was difficult to verify since these test strains produced little or no antibacterial activity in MRS broth, it can be concluded that the antibacterial substances produced by strain No. 1, No. 11 and CBLb2 are bacteriocins.

These bacteriocin-producing strains were found among 20 LAB strains isolated from meats. This incidence rate was found comparable with other studies (Garver and Muriana, 1993; Ahn and Stiles, 1990). Although these strains did not produce a detectable amount of bacteriocin in liquid medium, they did show significant inhibition against certain indicator strains on agar medium. The size of inhibition zones was comparable or even bigger than those from strains which produced a significant amount of bacteriocin in culture broth (Schillinger and Lucke, 1989). Therefore, it is quite possible for these bacteriocin-producing strains to dominate in meat systems, which are physically similar to conditions existing on agar media, by suppressing other bacteriocin-susceptible competitors.

4.4 Individual Inhibitory Factor Study

4.4.1. Strain Selection

Fourteen spoilage LAB strains were used for the selection of 2 strains from each species for further study of the impact of environmental factors on growth and viability of these groups of organisms. The LAB cultures were inoculated in modified MRS (MMRS) broth and incubated at 6°C anaerobically for 2 weeks at an initial inoculum of about 10^7 CFU/ml. The growth data (viable bacterial numbers) are plotted in Figure 16 (data listed in Appendix 6).

Except for *L. sake* strain No.9 which reached 10^8 CFU/ml after 2 weeks incubation, other strains did not show substantial increases in number (numbers stayed at the level of 10^7 CFU/ml). The final pH of the MMRS broth for all the LAB strains dropped from 6.0 to a range from 5.5 to 5.9 due to acid production. The pH dropped to about 5.0 for strain 9 (data not shown). Low pH may be one inhibitory factor influencing the extent of growth of these spoilage organisms. Since at 25°C these strains can easily reach 10^9 CFU/ml in MMRS at a very low inoculum (one colony), the incubation temperature (6°C) in this test may be another hurdle that affected the growth of these LAB. Another reason may be the depletion of the nutrients in the MMRS broth after 2 weeks' incubation.

No significant differences in the final cell number were found among the *Lc. mesenteroides* strains and *L. curvatus* strains, respectively. Therefore, two strains from each group (No.1 and No.11 for *Lc. mesenteroides*, No.10 and No.15 for *L. curvatus*) were randomly chosen for the individual factor study. In the *L. sake* group, the number of cells of strains No.14 and No.34 were substantially lower than No.6 and No.9, so these two latter strains were chosen.

4.4.2. Individual Factor Tests

4.4.2.1. NaCl

The separate growth data of the 6 selected strains in MMRS broth with 2.5%, 4%

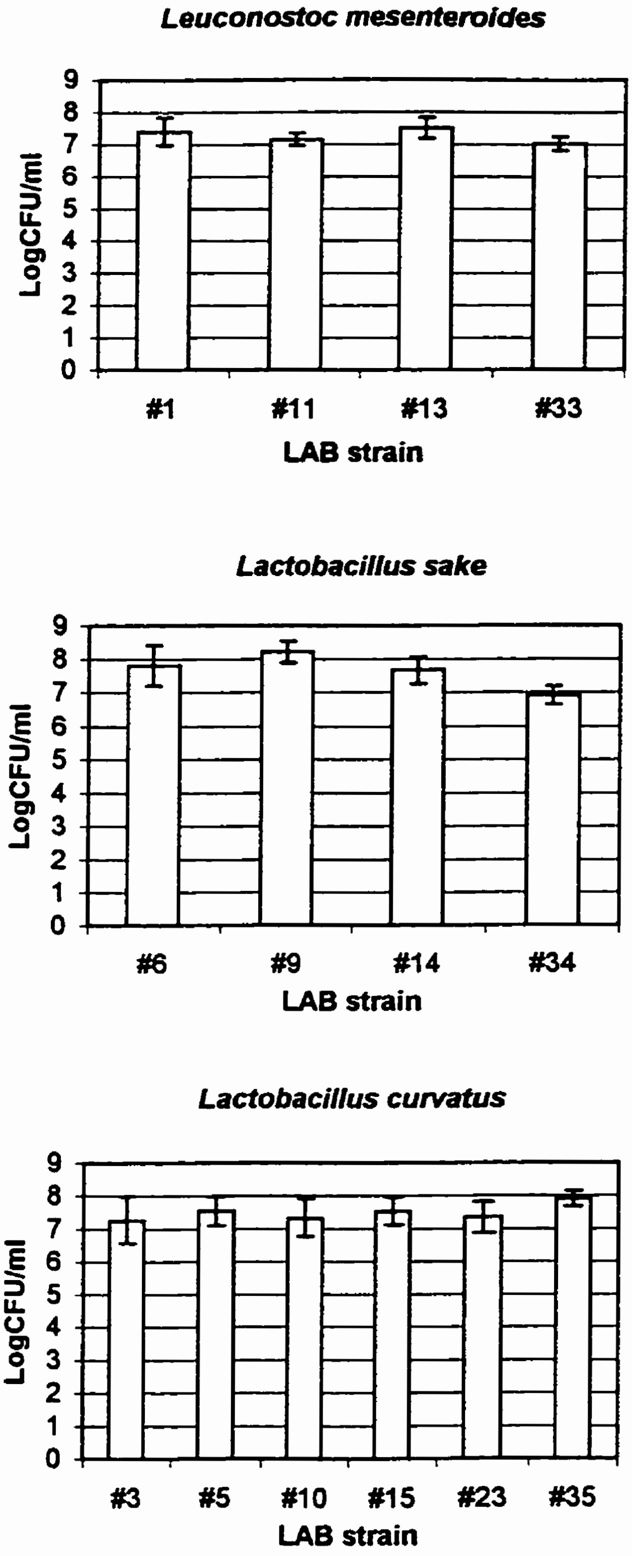


Figure 16. Growth ability of a group of spoilage LAB strains in MMRS broth incubated at 6°C for 2 weeks.

and 5.5% NaCl are presented in Figure 17 (numerical data are tabulated in Appendix 7). After 2 weeks' anaerobic incubation at 6°C, bacterial numbers stayed at the level of 10^7 CFU/ml when 2.5% NaCl was used. However, strain No.9 was able to reach about 10^8 CFU/ml. In contrast, when 4% and 5.5% NaCl were present in the MMRS broth, the inhibitory effect was quite significant. For strains No.1, No.11 and No.6, a 1-2 logCFU/ ml reduction was achieved.

For *Lc. mesenteroides* strains No.1 and No.11, a significant difference in growth ability was found between 2.5% NaCl and the higher concentrations. However, the final bacterial number was not significantly different between tests done in the presence of either 4% or 5.5% NaCl. The test results from the 2 *L. sake* strains was quite similar to results from the leuconostocs. It was found that strain No.6 was very sensitive to high levels of NaCl. About a 2 log CFU/ml reduction took place when the NaCl concentration was increased from 2.5% to 4%. However, for 4% and 5.5% NaCl, the number of cells of strain No.6 remained the same. NaCl had little effect on the growth of the *L. curvatus* strains No.10 and No.15. No significant difference was found among these 3 concentrations. However, it seemed No.15 grew better than No.10 with the bacterial number about one logCFU/ml higher at all 3 NaCl levels.

The final pH of the broth did not change much except in the 2.5% NaCl treatment, where the broth containing strain No. 9 dropped from pH 6.0 to about 5.0 (Figure 17). This was an indication of the active growth of this organism under these conditions. With the increase in bacterial number, a greater amount of acid was produced.

In general, it is well documented that spoilage LAB are not sensitive to NaCl at

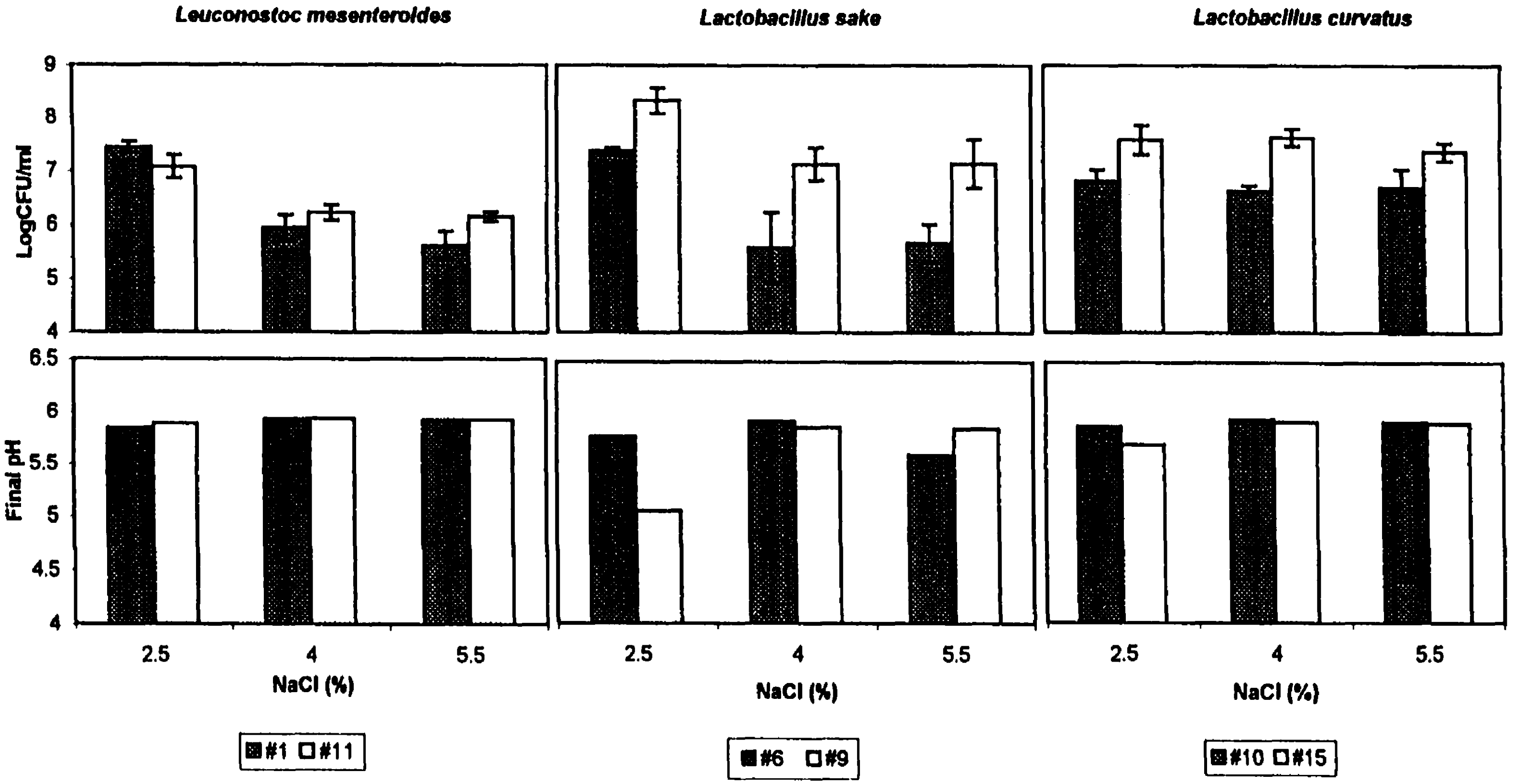


Figure 17. Effect of NaCl on the growth of spoilage LAB isolated from meats. About 10^7 CFU/ml of each culture was inoculated separately into MMRS broth (pH 6.0) containing 100 ppm $NaNO_2$ and incubated at 6°C anaerobically for 2 weeks.

intermediate pH and temperatures of 8°C and 12°C (Mol et al., 1971). However, there was a significant difference in the LAB numbers between 5°C and 8°C, when these organisms were incubated with 3.0-3.3% brine (Mol et al., 1971). Other than this report, there is little information about the growth of LAB with different NaCl levels at low temperatures such as 6°C. This low temperature might have contributed to the difference in growth ability of LAB at 2.5% NaCl and higher concentrations of NaCl.

Korkeala et al. (1990) studied the effect of NaCl on the growth capability of spoilage LAB. The delta absorbance (difference between the first and last absorbance reading), the gradient (slope of the logarithmic growth phase) and the lag phase (time delay until bacterial growth started) were calculated to characterize growth. However, this study was also carried out at ambient temperature and the length of the incubation was only about 50 h. It was found that *Lactobacillus* (*L. sake* and *L. curvatus*) strains were generally more resistant to NaCl than *Lc. mesenteroides* and *L. sake* was more halotolerant than *L. curvatus*.

In the present study, *L. curvatus* strains were shown to be more resistant to NaCl than *Leuconostoc* strains. However, the two *L. sake* strains (especially No.6) which were expected to be more NaCl tolerant, were quite sensitive to high levels of NaCl. It was found that the mode of inhibitory action of sodium nitrite on LAB can vary among different strains (Dodds and Collins-Thompson, 1984).This may also be the case with NaCl. It may be that No.6 was an exceptionally NaCl-sensitive *L. sake* strain. More importantly, the bacterial numbers of all the strains remained almost unchanged in the presence of 4% and 5.5% NaCl ($p < 0.05$). This means no additional inhibitory effect was achieved when the NaCl concentration was increased beyond 4%.

4.4.2.2. NaNO$_2$

The growth data of the 6 strains with 3 levels of NaNO$_2$ (100, 150 and 200 ppm, respectively) are presented in Figure 18 and are tabulated in Appendix 8. No significant reduction of bacterial numbers of test strains resulted from exposure to these concentrations of NaNO$_2$. For strains No.9 and No.15, a one logCFU/ml increase was achieved at all concentrations. There was no significant growth difference between *Leuconostoc* strains No.1 and No.11. For *L. sake* strains, No. 9 grew better than No.6. For *L. curvatus* strains, No.15 grew better than No.10. The test results in general were very consistent with the literature in that at intermediate pH, NaNO$_2$ had little affect on the growth of LAB unless a level of 400 ppm or higher was used (Korkeala et al., 1990).

It was found that there was little change in the final pH of the MMRS broth except for strain No.9, again, where the final pH dropped to 5.0-5.4. This was quite a reasonable change in view of the high number of bacteria in the broth.

It was observed that the average number of *L. sake* (at all 3 NaNO$_2$ levels) was slightly higher than that of *L. curvatus*, and the number of *L. curvatus* was slightly higher than found for *Lc. mesenteroides* strains. This was quite consistent with the results of Korkeala et al. (1990) but these differences were not statistically significant. Therefore, at permitted levels of sodium nitrite ($\leq$200pm), there is likely to be little inhibitory effect on the growth of spoilage LAB.

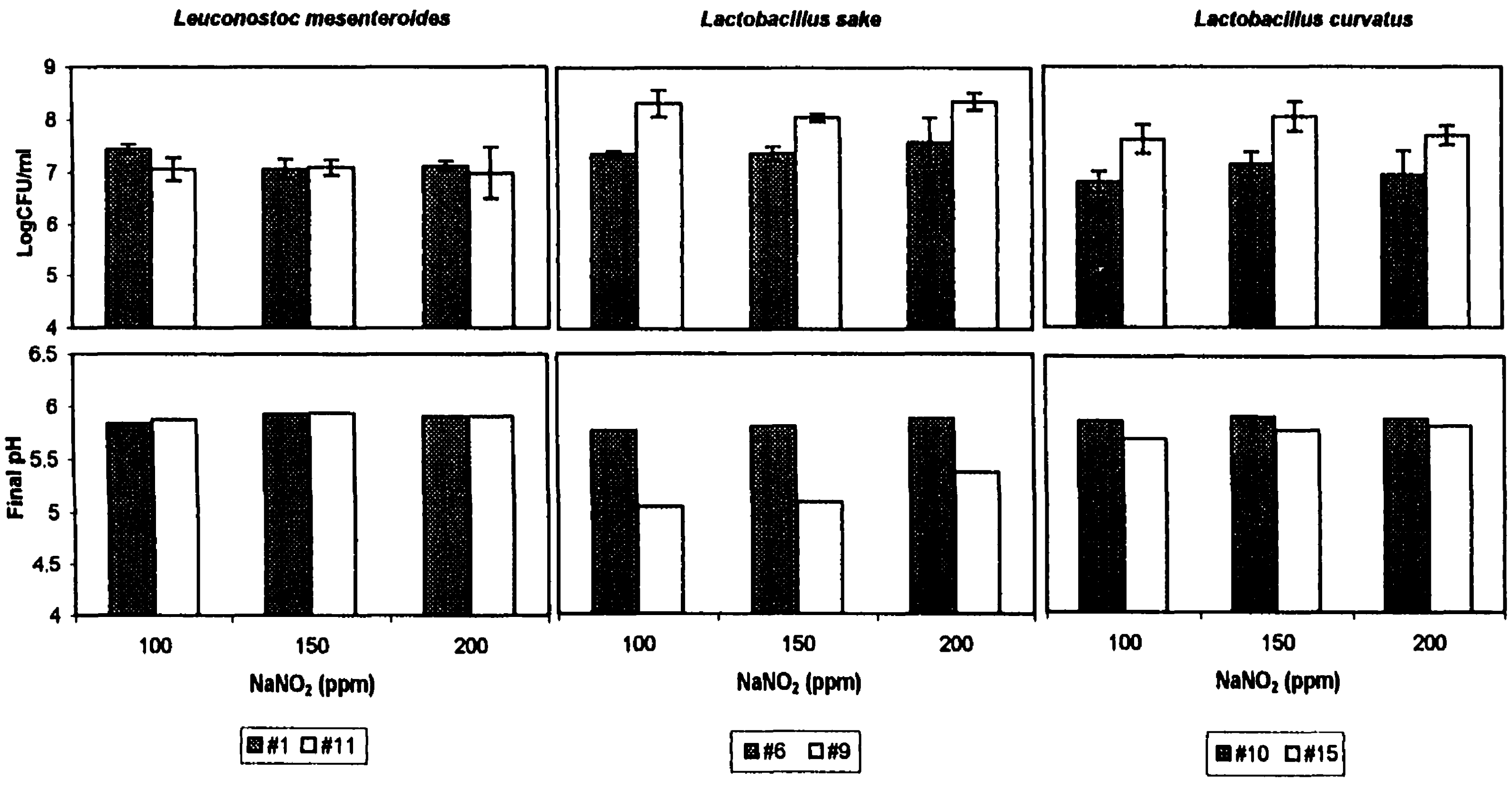


Figure 18. Effect of sodium nitrite on the growth of spoilage LAB from meats. About 10^7 CFU/ml of each culture was inoculated in MMRS broth (pH 6.0) containing 2.5% NaCl and incubated at 6°C anaerobically for 2 weeks.

4.4.2.3. Initial pH

The growth data of the 6 LAB strains with 3 different levels of initial pH (5.5, 6.0 and 6.5) are presented in Figure 19 (detailed data are found in Appendix 9). The growth patterns of the 3 different species in the presence of different initial pH were quite different. For the *Lc. mesenteroides* strains, compared with the initial inoculum there was a 0.5 logCFU/ml reduction for both strain No.1 and No.11 at an initial pH of 5.5. The bacterial number did not change significantly at pH 6.0. At pH 6.5, strain No.1 grew to about 10^8 CFU/ml and this was better than that shown by strain No.11. The bacterial number was significantly lower at pH 5.5 with each strain. For strain No.1, at the higher pH values, the increase in bacterial number was significant but no significant change in numbers was seen with strain No.11 between pH 6.0 and 6.5. The final pH of the broth did not decrease much (only 0.1-0.2 unit) for both strains at all 3 initial pH levels, which indicated that no excessive acid production took place.

For the *L. sake* strains, the growth pattern was very different from that of *Lc. mesenteroides*. With strain No.9, cell numbers increased significantly from pH 5.5 to pH 6.0, and then decreased significantly from 6.0 to 6.5. Strain No.6 shared the same growth pattern with No.9 except that the bacterial number was significantly lower at each pH level. It was also found that with an increase in the initial pH, the extent of the final pH drop also increased, however, there was no significant drop (0.2 unit) for strain No.6. However, for No.9, the pH dropped 0.37, 0.95 and 1.16 units at initial pH levels of 5.5, 6.0 and 6.5, respectively. From the size of the cell pellet formed at the bottom of the broth tube, it was estimated at pH 6.5, No.6 and No.9 could have achieved cell concentrations > 9 logCFU/ml,

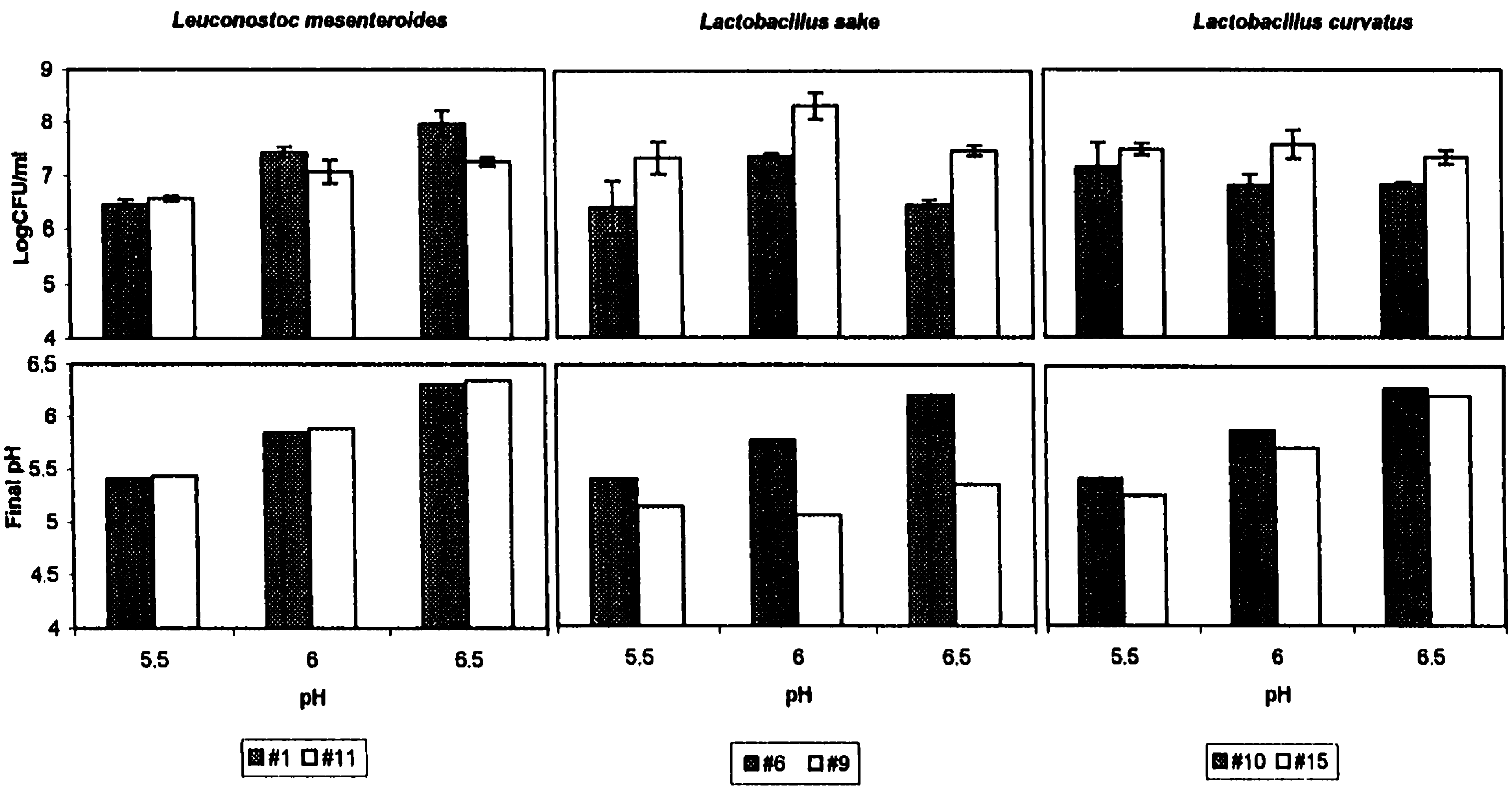


Figure 19. Effect of initial pH on the growth of spoilage LAB isolated from meats. About 10^7 CFU/ml of each culture was inoculated in standard MMRS broth and incubated at 6°C anaerobically for 2 weeks.

which would mean there were large numbers of dead cells in the broth. This might have been caused by the great amount of acid produced or by the depletion of growth nutrients in the MMRS broth. It was frequently found that strain No.6 often "killed" itself prematurely on MRS or APT agars incubated at 6°C or 25°C after one week. However, this may not happen in meats because meat is a highly buffered system and there are normally plenty of nutrients for spoilage LAB to utilize. Therefore, in meat inoculation tests, it is expected that *L. sake* strains will flourish and likely outgrow other competitors.

For *L. curvatus* strains, there was no significant difference in bacterial numbers among the 3 pH levels. This suggested that initial pH had little effect on the long-term growth of this species. Neither of the 2 strains reached 8 logCFU/ml at any initial pH level tested. Strain No.15 grew slightly better than No.10. There was about a 0.2 and 0.3 unit drop in pH for No.10 and No.15, respectively. Compared with the *L. sake* strains, this drop was not remarkable.

There is little information on the systematic study of the effect of initial pH on growth of spoilage LAB. Mol et al. (1971) studied the growth of *L. viridescens* LL6 at 8°C and 12°C in MRS broth containing 3.5% NaCl at various initial pH values. Without the addition of 200 ppm $NaNO_2$, pH had no effect on the growth of this organism. However, the presence of 200 ppm $NaNO_2$ decreased the growth rate markedly at pH 5.7, especially at 12°C. This interaction was also observed with *Lc. mesenteroides* and *L. sake* in the current study.

4.4.2.4. Temperature

The growth data of 6 spoilage LAB strains at 3 incubation temperatures (2°C, 6°C and 12°C) are presented in Figure 20 (detailed data are in Appendix 10). At 12°C, the inoculated MMRS both was incubated anaerobically one week to represent abusive product storage. At 6°C, the incubation time was 2 weeks to mimic the normal commercial condition at retail. At 2°C, the incubation lasted for 5 weeks to represent more desirable storage conditions. The growth patterns of these 3 species, again, were very different among different combinations of incubation time and temperature.

For *Lc. mesenteroides* strains No.1 and No.11, there was no significant difference in growth among different incubation temperatures, except that the number of No. 1 was significantly greater at 2°C than at 6°C or 12°C and this was probably due to the longer incubation time used at 2°C. Although there was no significant difference in numbers of cells between these two strains, No.1 seemed to grow slightly better. The final pH of the broth was slightly lower at 2°C and 6°C (0.2-0.3 unit) but was significantly lower at 12°C (0.5 unit). Maybe this was the reason that at 12°C, *Lc. mesenteroides* strains did not reach high numbers since they did not grow well at low pH (5.5), which had been shown in the previous pH tests.

L. sake strains grew well at 2°C and 6°C but there was a significantly lower number of viable cells of both strains No.6 and No.9 at 12°C. At the latter temperature a large quantity of what were probably dead cells was accompanied by a significant pH drop, especially with strain No.9. The final pH of the MMRS broth from No.9 dropped from 6.0 to 4.5 and it was estimated there were probably 10^9 - 10^{10} CFU/ml cells present at the peak of growth during incubation at 12°C. Interestingly, it was noted that there were comparable

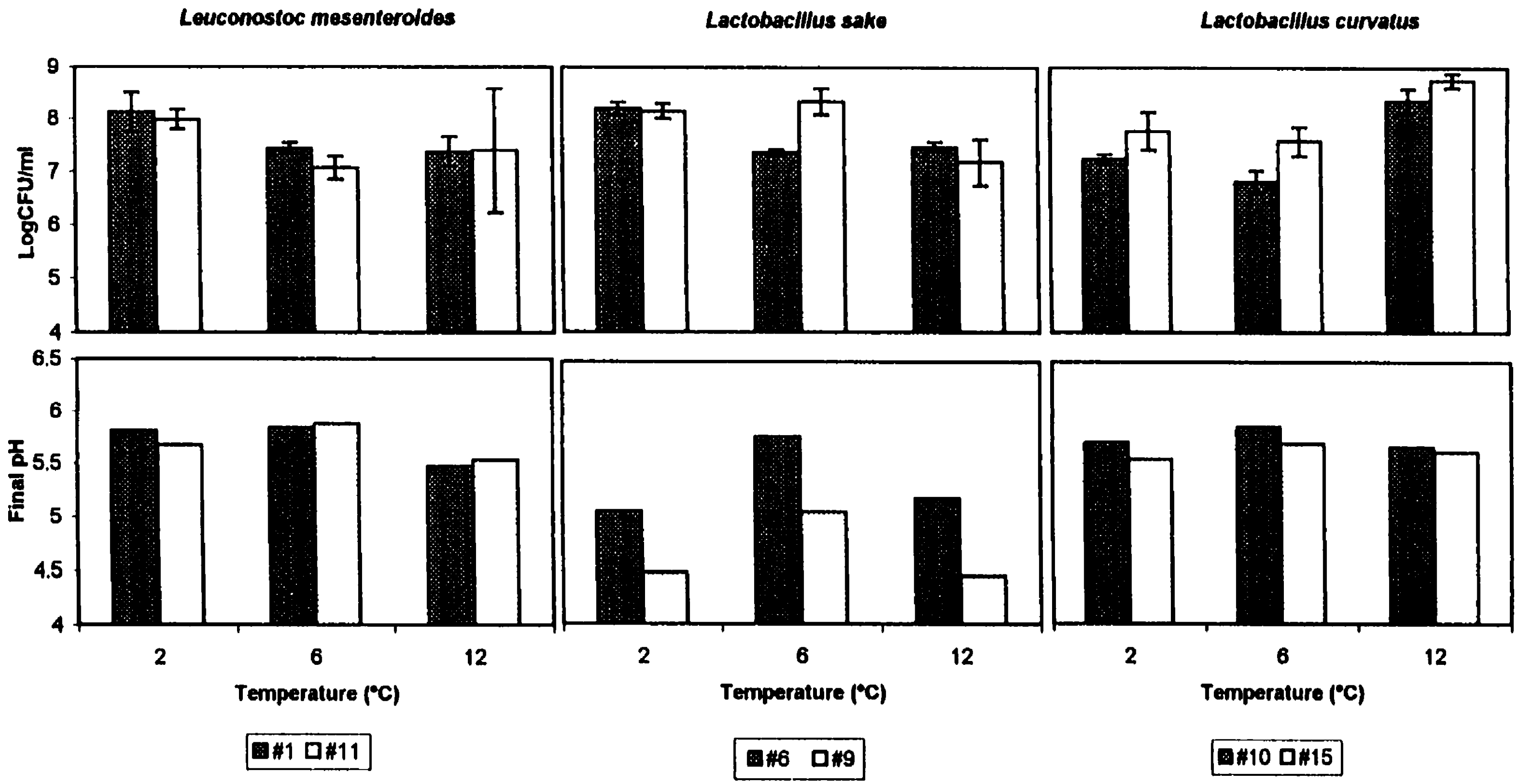


Figure 20. Effect of temperature on the growth of spoilage LAB isolated from meats. About 10^7 CFU/ml of each culture was inoculated in standard MMRS broth and incubated anaerobically at 12°C for one week, 6°C for 2 weeks or 2°C for 5 weeks.

pH drops at 2°C for both No.9 and No.6, where the bacterial numbers reached 10^8 CFU/ml. There are two possible explanations for this difference between 2°C and 12°C: (a) The rapid rate of pH drop may have had a remarkably inhibitory effect on *L. sake*; (b) A large number of *L. sake* cells died at 12°C, due to the depletion of nutrients from the MMRS broth. As discussed in the previous pH test, such a significant decrease in numbers of *L. sake* cells may not likely happen in meat systems due to their higher buffering capacity and relative abundance of nutrients.

For *L. curvatus* strains, there were significant differences in growth at the different temperatures, especially for strain No.10. At 2°C, the final bacterial numbers did not change much from their initial inoculum level. At 6°C, cell numbers were a little bit lower than at 2°C, where the incubation time was much longer. At 12°C, however, the bacterial numbers reached 10^8 - 10^9 CFU/ml, and this was an indication that the difference in growth rate between 2°C and 6°C was not as significant as that between 6°C and 12°C. The final pH of the broth did not drop remarkably at each of the 3 temperatures.

4.4.2.5 Summary

Two *Lc. mesenteroides* strains (No.1 and No.11), two *L. curvatus* strains (No.10 and No.15), and two *L. sake* strains (No.6 and No.9) were selected for individual inhibitory factor tests after screening growth data (viable cells) from 14 LAB strains by incubating them in standard MMRS broth (2.5% NaCl, 100 ppm $NaNO_2$, 1% sucrose, pH 6.0) anaerobically at 6°C for 2 weeks.

NaCl (>4%) had a significant inhibitory effect on the growth capability of *Lc.*

mesenteroides and *L. sake* strains at 6 °C but had no effect on *L. curvatus* strains. Sodium nitrite (≤ 200 ppm) had little effect on the growth of spoilage LAB strains in MMRS broth at 6 °C. Different incubation temperatures had a significant effect on the growth of all 3 species. It appeared that *L. curvatus* and *L. sake* grew faster at elevated temperature (12°C) than leuconostocs. The initial pH also had significant effect on *Lc. mesenteroides* and *L. sake* but had little effect on *L. curvatus*. At pH 5.5, lactobacilli grew better than leuconostocs. From Figures 17-20, it was also noted that there was a similar response for strains within the same species to the challenges of NaCl, $NaNO_2$, pH and temperature.

It was observed that at high initial pH (6.5) or "abusive" incubation temperature (12 °C), *L. sake* strains, especially No.9, suffered from what appeared to be self-induced lethality - lots of dead cells were found in the broth by visual estimation versus viable counts. It was estimated that cell numbers had probably reached 10^9 - 10^{10} CFU/ml at peak growth. Accompanying this phenomenon was a remarkable pH drop (> 1.0 unit) in the broth which indicated a large amount of acid was produced during growth.

Although the literature indicated that NaCl had little inhibitory effect on the growth of spoilage LAB (Mol et al., 1971; Korkeala et al., 1990), in the present study it was shown that the growth ability of *Lc.mesenteroides* and *L .sake* was significantly stronger in the presence of 2.5% NaCl than in 4.0% NaCl at 6 °C. However, except in dry fermented sausages which may have higher salt concentration (ca. 5%) in the final product, the normal NaCl concentration in vacuum-packaged cooked cured meats is about 2.5%. Therefore, NaCl at 2.5% plus varied incubation temperature and initial pH, which showed a substantial effect on the growth of spoilage LAB in the single inhibitory factor tests, were chosen as test variables in combination with 100 ppm $NaNO_2$ in the dominance tests for mixed cultures.

From the information obtained in the individual factor tests, it suggested that among these 3 groups of LAB, *L. sake* would dominate in cured meats at 6 or 12°C. At these temperatures, *L. curvatus* also would outgrow *Lc. mesenteroides*. At 2°C, all 3 groups grew well although it was generally believed that *Lc. mesenteroides* could not compete with lactobacilli at low temperatures. Maybe strain No.1 and No.11 are two low-temperature-tolerant *Leuconostoc* strains.

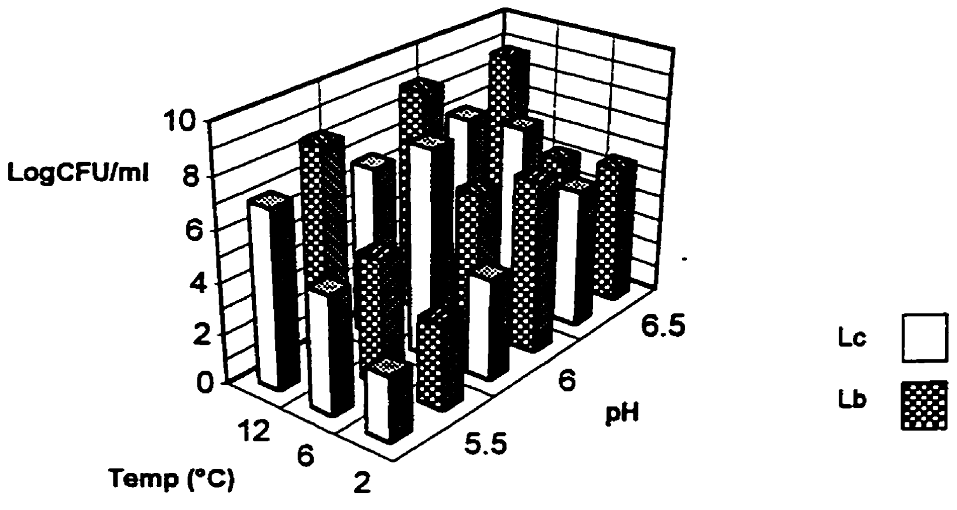
Based on their growth capability in the individual tests, *Lc. mesenteroides* strain No.1, *L. sake* strain No.9 and *L. curvatus* strain No.10 were chosen for the dominance test. Although strain No.15 grew better than No.10 at different temperatures and pH levels, No.10 was chosen for the mixed-culture test because it was sensitive to the bacteriocin produced by No.1 and it was thought that its use might bring some understanding about the role of bacteriocins in the dominance of spoilage LAB strains.

4.5 Dominance Test

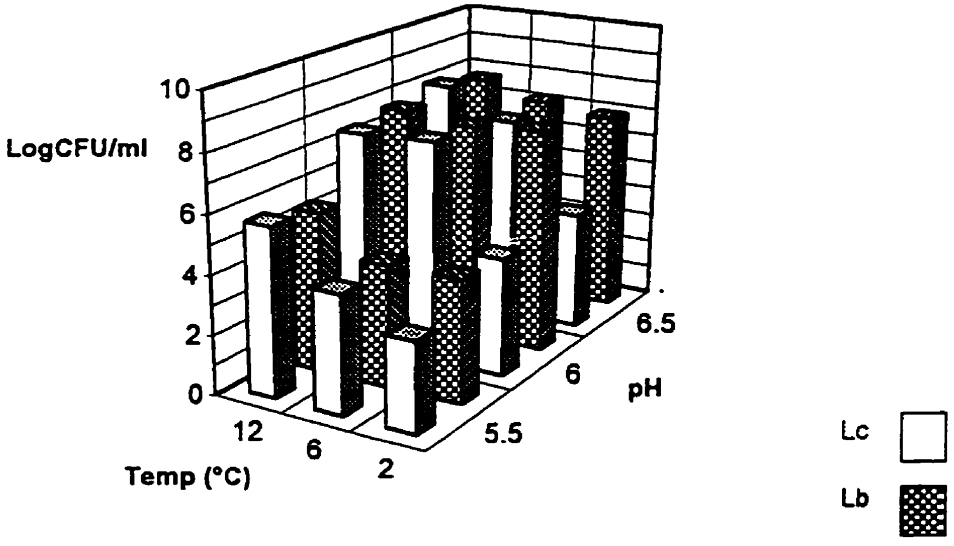
4.5.1. Dominance Tests With Equal Initial Ratio (10^4:10^4 CFU/ml)

The effects of initial pH and incubation temperature on *Lc. mesenteroides* strain No.1 and *L. curvatus* strain No.10 in mixed inocula at an initial ratio of 10^4:10^4 (CFU/ml) are presented in Figure 21A (detailed data are presented in Appendix 11).

When *Lc. mesenteroides* and *L. curvatus* were present at same initial level (10^4 CFU/ml), initial pH , temperature and their interaction had significant effects on their



A: Effects of pH and temperature on the growth of
Lc. mesenteroides* and *L. curvatus



B: Effects of pH and temperature on the growth of
Lc. mesenteroides* and *L. sake

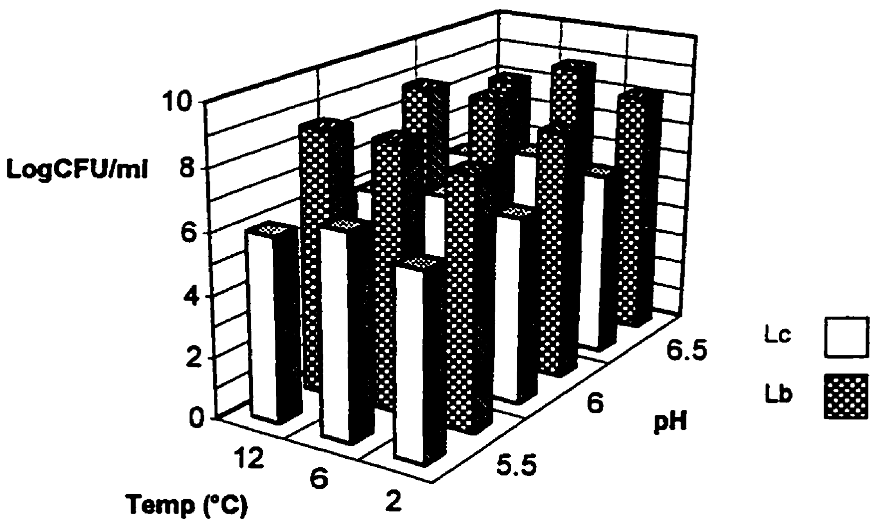
Figure 21. The effect of initial pH and incubation temperature on the growth of *Lc. mesenteroides* and *L. curvatus/sake* in mixed cultures at an equal initial ratio (10^4:10^4 logCFU/ml).Lc: *Lc. mesenteroides* ; Lb: *Lactobacillus* sp.

growth and dominance. When present in mixed cultures, *Leuconostoc* strain No.1 did not grow well at 2°C after 5 weeks' incubation (especially at pH 5.5), which was quite different from the result obtained in the individual inhibitory factor test. However, at 6°C, *Lc. mesenteroides* outgrew *L. curvatus* by 1-2 logCFU/ml. Just like in the individual factor tests, pH had little effect on the growth of *L. curvatus*. The numbers of *L. curvatus* reached 5 logCFU/ml at all 3 pH levels, which was only 1 logCFU/ml higher than the initial inoculum. At 12°C, pH had no effect on either No.1 or No.10. Strain No.10 reached 10^8~10^9 CFU/ml and No.1 was one or two logCFU/ml lower at all 3 pH levels. This indicated that for strains No.1 and No.10, temperature was more significant than pH in determining dominance. At 6°C, *Lc. mesenteroides* was more likely to dominate in the mixed culture while at 2°C and 12°C, *L. curvatus* was dominant. At 2°C with an initial pH of 5.5, both No.1 and No.10 were inhibited and viable cell numbers could be kept at 3 logCFU/ml if the initial bacterial load were low (4 logCFU/ml).

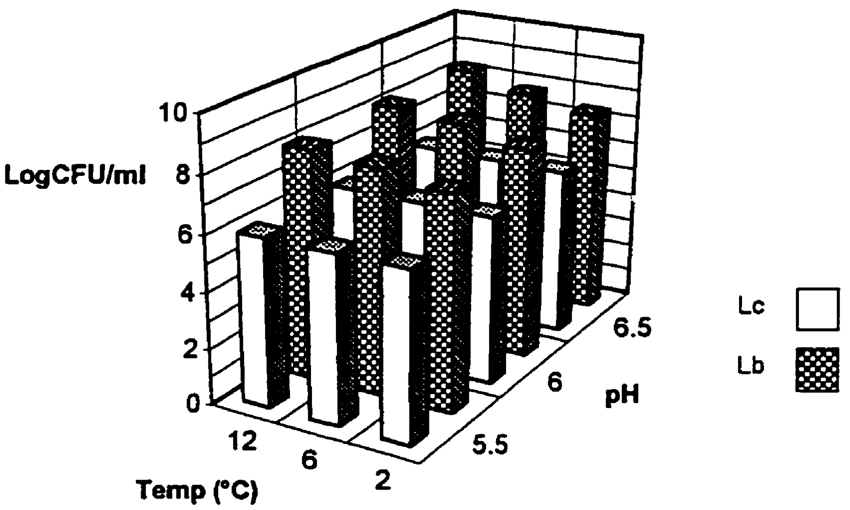
The growth patterns of strain No.1 and *L. sake* (No.9) at equal initial inoculum (10^4 CFU/ml) with different pH and temperature conditions are presented in Figure 21B (detailed data are recorded in Appendix 12). Again low temperature (2°C) had a substantial inhibitory impact on the growth of No.1. At all pH levels the cell number of strain No.1 remained constant or decreased ($\leq 10^4$ CFU/ml), resulting in the dominance of No.9. However, temperatures had little effect on the growth of *L. sake* strain No.9. At pH 5.5, both No.1 and No.9 grew poorly (10^3-10^5CFU/ml) but both of them reached 10^7~10^8 CFU/ml at pH 6.0 and 6.5 (except No.1 at 2°C). It was noted that in the dominance test, there was no self-inflicted mortality as happened to No.9 at 6°C and pH 6.5 (which was frequently observed in the individual inhibitory factor test). This might have been the result of competition of the mixed cultures for nutrients, which retarded the onset of peak growth of No.9 so that at the time of sampling, No.9 was still in a state of rapid cell division. Results showed that a combination of low initial pH (5.5)with low temperature (2°C) could be used as a possible means to extend the shelf life of vacuum-packaged cured meats if the initial bacterial load were low. Unfortunately, present day cured meat formulations have pH levels that range between 6.0 and 6.5.

4.5.2. Dominance Tests With Unequal Initial Ratio (10^7:10^4 or 10^4: 10^7 CFU/ml)

The effect of pH and temperature on the growth of *Lc. mesenteroides* strain No.1 and *L. curvatus strain* No.10 or *L. sake* strain No.9 at unequal inocula levels (10^7:10^4 or 10^4: 10^7 CFU/ml) are presented in Figure 22 and Figure 23 (detailed data are presented in Appendices 13-16). It was found that dominant bacteria always grew from the originally larger bacterial group. However, at 2°C when the initial number of *Leuconostoc* strain No.1 was larger, the final number of No.1 was less than 1 logCFU/ml higher than the *Lactobacillus* strains. This was caused by the sensitivity of No.1 to low temperature. In other cases, the initially dominant strain was 1-2 logCFU/ml higher than its opponent. At the end of the test, it was found very hard to enumerate the LAB strain with lower number on M5 plates when the other strain was ≥2 logCFU/ml higher. In some cases, for example, when No.9 reached 10^8 CFU/ml, a number - 6 logCFU/ml was recorded arbitrarily for No.1 because the level of No.1 was less than 10^6 CFU/ml and it was impossible to determine their numbers accurately.

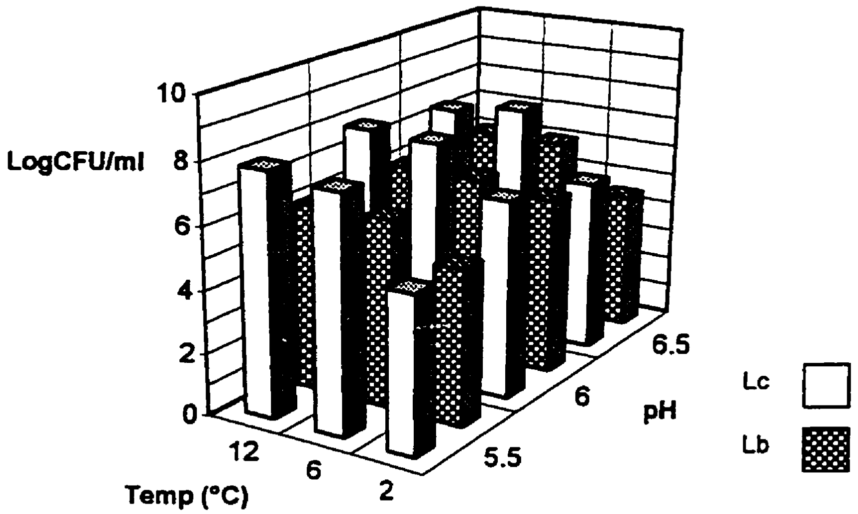


A: Effects of pH and temperature on the growth of *Lc. mesenteroides* and *L. curvatus*

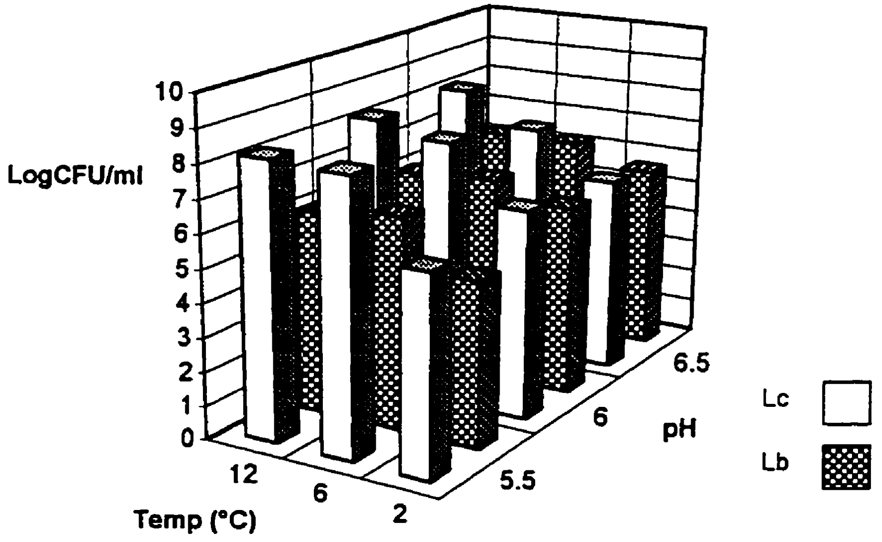


B: Effects of pH and temperature on the growth of *Lc. mesenteroides* and *L. sake*

Figure 22. The effect of initial pH and incubation temperature on the growth of *Lc. mesenteroides* and *L. curvatus/sake* in mixed cultures at unequal initial ratio (10^4:10^7 logCFU/ml). Lc: *Lc. mesenteroides*; Lb: *Lactobacillus* sp.



A: Effects of pH and temperature on the growth of *Lc. mesenteroides* and *L. curvatus*



B: Effects of pH and temperature on the growth of *Lc. mesenteroides* and *L. sake*

Figure 23. The effect of initial pH and incubation temperature on the growth of *Lc. mesenteroides* and *L. curvatus/sake* in mixed cultures at unequal initial ratio (10^7:10^4 logCFU/ml). Lc: *Lc. mesenteroides*; Lb: *Lactobacillus* sp.

Lactobacillus strains No.9 and No.10 were sensitive to the bacteriocin produced by *Leuconostoc* strain No.1, but there was no substantial inhibitory effect observed in this mixed-culture study (Figure 22), although there was evidence that in some cases the presence of bacteriocin-sensitive bacteria in broth may significantly enhance the bacteriocin production by the bacteriocin-producing strain (Sip et al., 1998). Nonetheless, our earlier inability to detect bacteriocin production by producer strains in broth media may mean that model experiments where broth is used to test for dominance are biased and insensitive to the true influence of bacteriocins in determining dominance on solid substrates.

4.5.3. Summary

When present in mixed cultures, *Leuconostoc* strains did not grow well at 2°C and an initial pH of 5.5, compared with lactobacilli. At normal pH (6.0 and 6.5) and higher temperatures (6°C and 12°C), dominant bacteria always grew from the originally larger bacterial group. When leuconostocs and lactobacilli were present in equal initial numbers, lactobacilli dominated at 2°C and pH 5.5. In the *Lc. mesenteroides* and *L. curvatus* system, *Lc. mesenteroides* dominated at 6°C ($pH \geq 6.0$) while *L. curvatus* dominated at 12°C ($pH \geq 6.0$). In the *Lc. mesenteroides* and *L. sake* system, there was no significant differences in number between *Lc. mesenteroides* and *L. sake* (temperature ≥ 6°C , $pH \geq 6.0$).

4.6. Differential Enumeration of Bacterial Strains by PFGE

An M5 plate prepared from a mixed MRS broth containing 3 different LAB strains (No.3, 10 and 13) and having about 100 colonies was enumerated. The number of blue colonies (*L. curvatus* strains No.3 plus No.10) was 98 and the number of white colonies, which were *Leuconostoc* strain No.13, were found to be 18. Using Harrison's disc (Page 55, Chapter 3), 10 colonies were randomly chosen for this modified PFGE analysis. The gel image of PFGE analysis from this differential enumeration experiment is presented in Figure 24.

In this analysis, a blank agarose gel slice (control) was cut 1/3 smaller in size for easy recognition when loaded to the wells. Two blank slices were loaded at designated wells (Lane B and B'). Other digested DNA slices were randomly loaded into the other wells. From Figure 24, it was easy to determine that Lane F', D' and C' were strain No.10, Lane E', A', A, E and F were strain No.3 and Lane C and D were strain No.13, when compared with the reference result (Figure 9). Therefore, the ratio of strains No.13, No.10 and No.3 was 2:3:5.

Then calculation of the number of each strain on the M5 plate was completed given that the total number present was 116 colonies (98+18). It was estimated that there were 23 colonies of No.13 (116×2/(2+3+5)), 35 colonies of No.10 (116×3/(2+3+5)) and 58 colonies of No.3 (116×5/(2+3+5)). Compared with the results obtained with differential enumeration by color (18 vs. 98), the estimated number for No.13 and lactobacilli, respectively, by PFGE was quite close (23 vs. 93). However, what is unique from the PFGE method is that the 2 different strains of *L. curvatus* can be differentially enumerated as well.

In microbiological research, sometimes it is necessary to know the number of each strain in a mixed culture. For example, in order to take a look at the effect of *in situ*-produced bacteriocin on a bacteriocin-sensitive strain, it is necessary to track the growth of

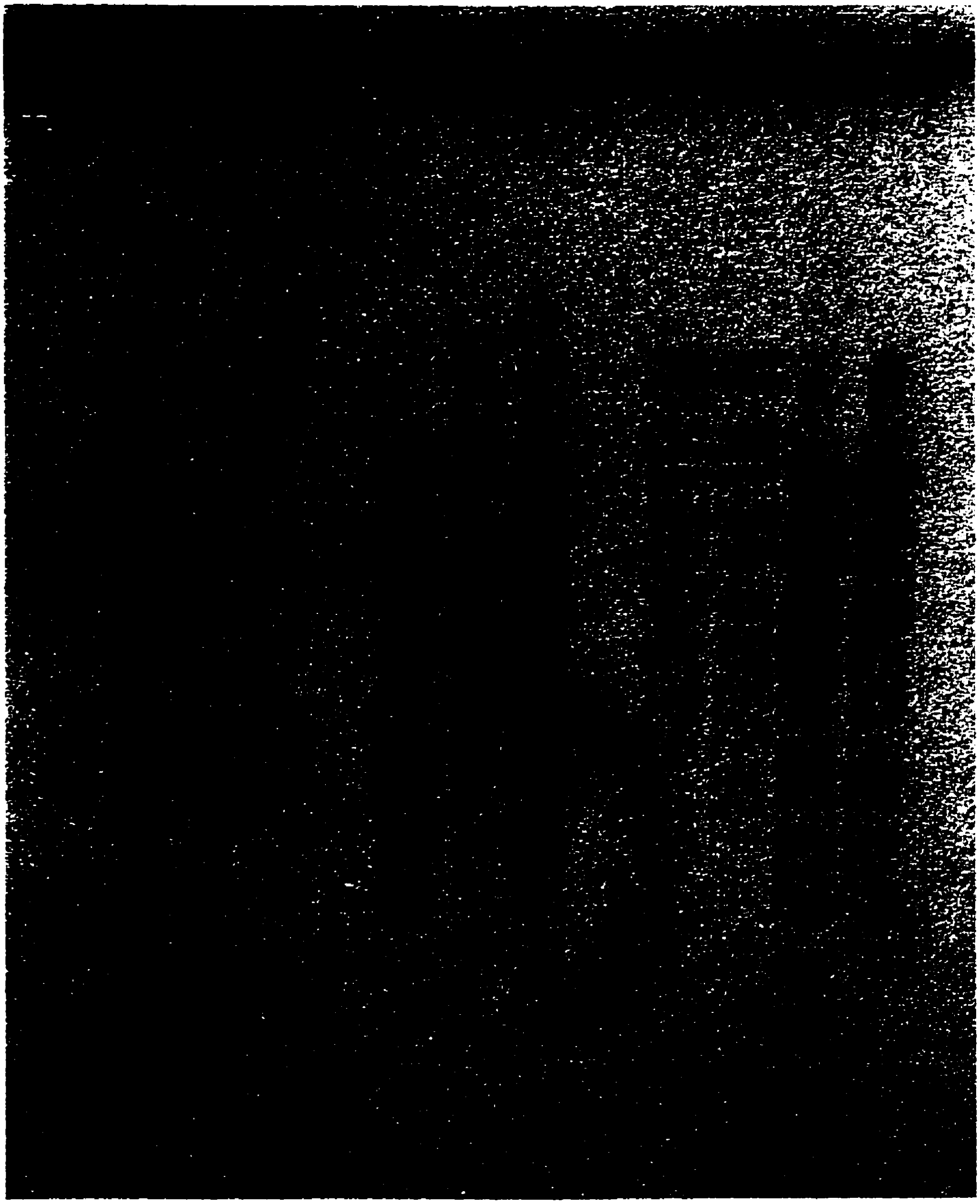


Figure 24. PFGE patterns of the genomic DNA digested with *Sma*I for strain No.3, 10, 13 to validate the newly developed PFGE differential enumeration method. The central lane marked with "M" is the size standard. The unit of the numbers is kb. Blank agarose gel slices were intentionally loaded in Lanes B and B'.

each of two strains. Lactic acid bacteria are a group of microorganisms that share a lot of growth requirements and biochemical similarities. Usually it is difficult to find a suitable medium for differential enumeration, especially for the strains within the same genus or species. The methods commonly used for designing selective or differential media are based on the observation of differences in antibiotic resistance and sugar fermentation. However, these methods can only by applied to those organisms possessing unique biochemical properties. Nissen et al., (1996) developed a method for differential enumeration of *Carnobacterium* spp. and *Leuconostoc* spp. by rRNA probes labeled with ^{32}P. However, these probes were only genus-specific and it was not possible for strain identification within genus or species.

PFGE is a powerful genotyping method for bacterial strain characterization. This newly developed method can also be utilized to provide a universal method for differential enumeration of bacterial strains in mixed cultures. Using this method, more than 2 strains can be enumerated from a single mixture, which is another advantage over conventional differential media. The key innovation of this method is the simultaneous digestion procedure during which, a group of slices, each containing DNA from different colonies on the same agar plate, were mixed together in one Ependorff tube and the digestion was done in this single tube. This procedure saved a considerable amount of time, energy and cost. Since the analysis of one plate needs only one tube, several plates can be analyzed together to promote efficiency. After digestion, slices can be loaded in the wells randomly because one is only interested in the electrophoresis pattern and the number of identical lanes in the gel image, not the order of the lanes. The control test (Figure 24) verified that the "composite-simultaneous" method did not affect the proper digestion of the DNA in each slice. The DNA-free agarose gel slices remained clean and clear in the PFGE analysis, which indicated that no DNA fragments caused cross-contamination. The reason is quite obvious: the DNA fragments (kb) were immobilized in the agarose gel matrix and could not easily move around.

Another novel aspect of this technique is the direct incorporation of bacterial cells from individual colonies into low melting point (LMP) agarose. This involved growing cells to a colony size of 0.5-1.0 mm in diameter (to obtain enough DNA material for PFGE analysis) and subsequently dispensing the cell cluster directly into 40°C LMP agarose gel (1%).

This PFGE differential enumeration technique is a reliable method because in most cases the DNA restriction pattern does not change when the bacteria encounter various physical or chemical challenges. Although it is still an estimation (not direct counting), it does provide informative results that can not be achieved by conventional methods. This procedure will be very useful in future work when the dominance patterns of strains within the same genus or species are investigated and also can be extended to research on other biological species.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

Results of the microbial analysis of the vacuum-packaged sliced ham and corned beef samples revealed that both homofermentative lactobacilli and leuconostocs were present in the spoiled meat and the number of these LAB reached 10^7-10^9 CFU/cm^2 on the meat surface. The number of homofermentative lactobacilli was a little bit higher than leuconostocs but no more than one order. The organisms that formed ropy slime on the corned beef samples lost their ropy slime-producing capability on agar plates. The bacterial number in the slime was one to two orders higher than on the meat surface. Compared with the control sample, the addition of sucrose to the corned beef samples did not change the dominance pattern of the LAB since the later API tests indicated that all LAB strains isolated from corned beef samples were able to utilize sucrose anaerobically.

Data indicated that APT, MRS, MRSD and M5 media were all suitable for the recovery and cultivation of LAB isolates. The color (blue or white) of the colonies on M5 agar sometimes was not stable and appeared to be strain dependent. Incubation of M5 plates at 25°C anaerobically for 36 h was found suitable for clear differentiation of LAB strains under study.

From the morphological characteristics, key biochemical reactions and the results of the API 50 CHL tests, the strains isolated from sliced ham samples were identified as *Lactobacillus curvatus* and *Leuconostoc mesenteroides* ssp. *mesenteroides* strains, and the isolates from the corned beef samples were identified as *Lactobacillus sake* and *Leuconostoc mesenteroides* ssp. *mesenteroides* strains, respectively. Pulsed-field gel electrophoresis (PFGE) of the LAB genomic DNA with *Sma*I digestion distinguished all the LAB strains under study. Some isolates from the meat samples which shared identical API profiles were identified as one strain after the PFGE analysis. Different strains possessed different PFGE profiles. No strains under study shared very close similarity such as 2-3 band differences in their macrocleavage patterns, and this indicated that these strains are not genetically closely related to each other. However, a RESTML analysis of the PFGE patterns of 7 *Leuconostoc* strains revealed that two bacteriocin-producing strains (No.1 and No.11) both isolated from Quebec had closer a relationship than the others.

The sizes of the DNA fragments digested with *Sma*I for *Lc. mesenteroides* ranged from 230 kb to 10 kb and the size range for *L. curvatus* and *L. sake* was from 110-130 kb to 6 kb. It was easy to differentiate *Leuconostoc* and *Lactobacillus* spp. by direct comparison of their PFGE gel patterns. However, differentiation of *L. curvatus* and *L. sake* by PFGE only was problematic since these two species shared similar digested DNA size range.

A "composite-simultaneous" restriction endonuclease digestion method was developed which enabled differential enumeration of LAB strains by PFGE.

Among 20 LAB strains, two *Lc. mesenteroides* strains (No.1 and No.11) and one *L. sake* strain (CBLb2) were able to produce bacteriocin and two *L. sake* strains (No.6 and No.9) and one *L. curvatus* strain (No.10) were found to be sensitive to these bacteriocins. As reported in other studies, these bacteriocin-producing bacteria could only produce bacteriocin on the agar surface.

After screening growth data(viable cells), two strains from each species (*L. curvatus* and *L. sake*) were selected for individual inhibitory factor tests. Low concentrations of $NaNO_2$ (<200 ppm) had little effect on the growth of spoilage LAB strains in MMRS broth at 6°C. High levels of NaCl (> 4%) had a significant inhibitory effect on the growth capability of *Lc. mesenteroides* and *L. sake* strains at 6°C but had no effect on *L. curvatus* strains. The initial pH of the MMRS broth also had significant effect on *Lc. mesenteroides* and *L. sake* but had little effect on *L. curvatus*. Lactobacilli grew better than *Lc. mesenteroides* at pH 5.5. Different incubation temperatures had a significant effect on the growth of all 3 species. It appeared that *L. curvatus* and *L. sake* grew faster at 12°C than *Lc. mesenteroides*. It was also noted that there was a similar response for strains within the same species to the challenges of NaCl, $NaNO_2$, pH and temperature.

Based on their growth capability in the individual tests, *Lc. mesenteroides* strain No.1, *L. sake* strain No.9 and *L. curvatus* strain No.10 were chosen for dominance tests. When present in mixed cultures, the *Leuconostoc* strain did not grow well at 2°C and initial pH 5.5, compared with lactobacilli. At normal pH (6.0 and 6.5) and higher temperatures (6°C and 12°C), dominant bacteria always grew from the originally larger bacterial group. When leuconostocs and lactobacilli were present in equal initial numbers, lactobacilli dominated at 2°C and pH 5.5. In the *Lc. mesenteroides* and *L. curvatus* system, *Lc. mesenteroides* dominated at 6°C ($pH \geq 6.0$) while *L. curvatus* dominated at 12°C ($pH \geq 6.0$). In the *Lc. mesenteroides* and *L. sake* system, there were no significant differences in number between *Lc. mesenteroides* and *L. sake* (temperature≥6°C , $pH \geq 6.0$). No substantial inhibitory effect of bacteriocin was observed in the mixed cultures.

Recommendations for future research based on this study would be:

- Search for a suitable method to break up the chains of leuconostocs without producing cell injury for their accurate enumeration on agar plates.
- Search for an antibiotic-based selective medium for lactobacilli or leuconostocs in mixtures since M5 is not suitable for successful enumeration when there is more than 2 logCFU/ml difference between the numbers of these two genera.
- In order to save time and cost in future strain isolation and identification processes PFGE analysis should be conducted ahead of the API tests since strains that have an identical PFGE pattern are not likely to show any difference in the API test.
- In further PFGE study of *L. curvatus* and *L. sake*, multi-enzyme restriction analysis plus phylogenetic study (e.g. RESTML) should be done to clearly differentiate these two species.
- To avoid the depletion of nutrients in MMRS broth during the long-term incubation, double-strength broth could be used. Continuous observation of LAB growth should be carried out to characterize the growth rate and growth peak during the challenge of each inhibitory factor.
- Investigation of strain dominance in a meat system should be carried out to validate the results obtained in the *in vitro* tests. The effect of bacteriocin production should be re-evaluated since meat surfaces are physically similar to conditions existing on agar media. The PFGE differential enumeration method can be used in multi-strain inoculation studies.

References

Ahn, C. and M. E. Stiles. 1990. Antibacterial activity of lactic acid bacteria isolated from vacuum-packaged meats. J. Appl. Bacteriol. 69: 302-310.

Ahvenainen, R., Kivikataja, R. -L. and E. Skytta. 1990. Factors affecting the shelf-life of gas and vacuum-packed cooked meat products. Part II: Vienna sausages. Lebensm. Wiss. Technol. 23: 130 - 138.

Allen, J. R. and E. M. Foster. 1960. Spoilage of vacuum-packaged sliced processed meats during refrigerated storage. Food Res. 25: 19 - 25.

Bellengier, P., Richard, J. and C. Foucaud. 1997. Associative growth of *Lactococcus lactis* and *Leuconostoc mesenteroides* strains in milk. J. Dairy Sci. 80: 1520 - 1527.

Bell, R. G. and C. O. Gill. 1982. Microbial spoilage of luncheon meat prepared in an impermeable plastic casing. J. Appl. Bacteriol. 53: 97 - 102.

Charteris, W. P. Kelly, P. M., Morelli, L. and J. K. Collins. 1997. Selective detection, enumeration and identification of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in mixed bacterial populations. Int. J. Food Microbiol. 35: 1 - 27.

Bjorkroth, J., and H. J. Korkeala. 1996. Evaluation of *Lactobacillus sake* contamination in vacuum-packaged sliced cooked meat products by ribotyping. J. Food Prot. 59: 398 - 401.

Bjorkroth, J., Ridell, J. and H. Korkeala. 1996. Characterization of *Lactobacillus sake* strains associating with production of ropy slime by randomly amplified polymorphic DNA (RAPD) and pulsed-field gel electrophoresis (PFGE) patterns. Int. J. Food Microbiol. 31: 59-68.

Brooks, J., Haines, R. B., Moran, T. and J. Pace. 1940. The function of nitrate, nitrite and bacteria in curing bacon and hams. Dept. Sci. Ind. Research, Food Invest. Board. Special Rep. 49, London.

Borch, E. and E. Nerbrink. 1989. Shelf-life of emulsion sausages stored in vacuum or modified atmospheres. Proc. 35th International Congress of Meat Science and Technology, Copenhagen. Vol. III, pp. 470 - 477.

Borch, E., Kant-Muermans, M. -L. and Y. Blixt. 1996. Bacterial spoilage of meat and cured meat products. Int. J. Food Microbiol. 33: 103 - 120.

Buncic, S., Avery, S. M. and S. M. Moorhead. 1997. Insufficient antilisterial capacity of low inoculum *Lactobacillus* cultures on long-term stored meats at 4°C. Int. J. Food. Microbiol. 34: 157 - 170.

Carolissen-Mackay, V., Arendse, G. and J. W. Hastings. 1997. Purification of bacteriocins of lactic acid bacteria: problems and pointers. Int. J. Food Microbiol. 34: 1 - 16.

Castellani, A. G. and C. F. Niven. 1955. Factors affecting bacterio-static action of $NaNO_2$. J. Appl. Microbiol. 3: 154 - 159.

Condon, S. 1987. Responses of lactic acid bacteria to oxygen. FEMS Microbiol. Rev. 46: 269 - 280.

Dodds, K. L. and D. L. Collins-Thomson. 1984. Nitrite tolerance and nitrite reduction in lactic acid bacteria associated with cured meat products. Int. J. Food Microbiol. 1: 163-170.

de Man, J. C. Rogosa, M and M. E. Sharpe. 1960. A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. 23: 130 - 135.

Donnelly, L. S., Ziegler, G. R. and J. C. Acton. 1982. Effect of liquid smoke on the growth of lactic acid starter cultures used to manufacture fermented sausage. J. Food Sci. 47: 2074 - 2075.

Dykes, G. A. and A. von Holy. 1994. Taxonomic status of atypical *Lactobacillus sake* and *Lactobacillus curvatus* strains associated with vacuum-packed meat spoilage. Curr. Microbiol. 28: 197 - 200.

Dykes, G. A., Britz, T. J. and A. von Holy. 1994. Numerical taxonomy and identification of lactic acid bacteria from spoiled, vacuum-packed Vienna sausages. J. Appl. Bacteriol. 76: 246 - 252.

Dykes, G. A., Cloete, T. E. and A. von Holy. 1995. Taxonomy of lactic acid bacteria associated with vacuum-packed processed meat spoilage by multivariate analysis of cellular fatty acids. Int. J. Food Microbiol. 28: 89 - 100.

Egan, A. F. and T. A. Roberts. 1987. Microbiology of meat and meat products. *in* Essays in Agricultural and Food Microbiology. (Norris, J. R. and Pettipher, G. L. eds.) pp. 167 - 197. G. L. John Wiley and Sons Ltd., New York.

Entis, P. and P. Boleszczuk. 1986. Use of fast green FCF with tryptic soy agar for aerobic plate count by the hydrophobic grid membrane filter. J. Food Prot. 49: 278 - 279.

Erdman, A. M., Watts, B. M., and L. C. Ellias. 1954. Smoke flavor and ascorbic acid as preservatives for fatty fish. Food Technol. 8: 320 - 323.

Evans, J. B. and C. F. Niven. 1951. Nutrition of the heterofermentative lactobacilli that cause greening of cured meat products. J. Bacteriol. 62: 599 - 603.

Farber, J. M. 1996. An introduction to the hows and whys of molecular typing. J. Food Prot. 59: 1091-1101.

Felsenstein, J. 1992. Phylogenies from restriction sites: a maximum-likelihood approach. Evolution, 46: 159-173.

Finegold, S. M. and W. J. Martin. 1982. Bailey and Scott's Diagnostic Microbiology. pp. 671. The C. V. Mosby Company, St. Louis, USA.

Foegeding, P. M., Thomas, A. B., Pilkington, D. H. and T. R. Klaenhammer. 1992. Enhanced control of *Listeria monocytogenes* by *in situ*-produced pediocin during dry fermented sausage production. Appl. Environ. Microbiol. 58: 884 - 890.

Franz, C. M. A. P. and A. von Holy. 1996. Thermotolerance of meat spoilage lactic acid bacteria and their inactivation in vacuum-packed Vienna sausages. Int. J. Food Microbiol. 29: 59 - 73.

Garriga, M. Hugas, M., Gou, P., Aymerich, M. T. Arnau, J. and J. M. Monfort. 1996. Technological and sensorial evaluation of *Lactobacillus* strains as starter cultures in fermented sausages. Int. J. Food Microbiol. 32: 173 - 183.

Garver, K. I. and P. M. Muriana. 1993. Detection, identification and characterization of bacteriocin-producing lactic acid bacteria from retail food products. Int. J. Food Microbiol. 19: 241 - 258.

Garvie, E. I. 1986. Genus *Leuconostoc* van Tieghem 1878. *in* Bergey's Manual of Systematic Bacteriology. (Sneath, P. H. A., Mair, N. S., Sharpe, M. E. and Holt, J. G. eds.) Vol. 2. pp. 1071 - 1075. Williams and Wilkins, Baltimore, MD.

Geis, A., Singh, J. and M. Teuber. 1983. Potential of lactic streptococci to produce bacteriocin. Appl. Environ. Microbiol. 45: 205-211.

Gill, C. O., Friske, M., Tong, A. K. W. and J. C. McGinnis. 1995. Assessment of the hygienic characteristics of a process for the distribution of processed meats, and of storage conditions at retail outlets. Food Res. Int. 28: 131 - 138.

Gregersen, T. 1978. Rapid method for distinction of Gram-negative from Gram-positive bacteria. European J. Appl. Microbiol. Biotechnol. 5: 123 - 127.

Harrigan, W. F. and M. E. McCance. 1976. Laboratory Methods in Food and Dairy Microbiology. Academic Press, London, 452 pp.

Hill, M. J. 1991. Nitrates and nitrites from food and water in relation to human disease. *in* Nitrates and Nitrites in Food and Water. (Morton, I. D. ed.) pp. 171-175. Ellis Horwood. London.

Hitchener, B. J. Egan, A. F. and P. J. Rogers. 1982. Characteristics of lactic acid bacteria isolated from vacuum packed beef. J. Appl. Bacteriol. 52: 31 - 37.

Holley, R. A. 1997. Asymmetric distribution and growth of bacteria in sliced vacuum-packed ham and bologna. J. Food Prot. 60: 510 - 519.

Holley, R. A. and G. E. Millard. 1988. Use of MRSD medium and the hydrophobic grid membrane filter technique to differentiate between pediococci and lactobacilli in fermented meat and starter cultures. Int. J. Food Microbiol. 7: 87 - 102.

Holley, R. A., Lamoureux, M. and F. Dussault. 1996. Identification of lactic acid bacteria from vacuum-packed cooked luncheon meat and induction of repairable injury by mild thermal stress. Lebensm. Wiss. Technol. 29: 114 - 122.

Holley, R. A., Doyon, G. Fortin, J. Rodrigue, N. and Carbonneau, M. 1996. Post-process, packing induced fermentation of delicatessen meats. Food Res. Int. 29: 35 - 48.

Holzapfel, W. H. and E. S. Gerber. 1986. Predominance of *Lactobacillus curvatus* and *Lactobacillus sake* in the spoilage association of vacuum-packaged meat products. pp. 26. Abstracts of the 32nd European Meeting of Meat Research Workers, Ghent.

Hugas, M., Garriga, M., Aymerich, T. and J. M. Monfort. 1993. Biochemical characterization of lactobacilli from dry fermented sausages. Int. J. Food Microbiol. 18: 107 - 113.

Instruction manual. 1993. pp. 4 - 6. API 50 CH system. BioMerieux SA, Marcy-l'Etoile, France.

Kandler, O. 1983. Carbohydrate metablism in lactic acid bacteria. Antonie von Leuwenhoek, 49: 209 - 224.

Kandler, O. and N. Weiss. 1986. Regular, nonsporing Gram-positive rods. *in* Bergey's Manual of Systematic Bacteriology. (Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt, J.G. eds.) Vol. 2. pp. 1208-1234. Williams and Wilkins, Baltimore, MD.

Kelly, W. J., Asmundson, R. V., Harrison, G. L. and C. M. Huang. 1995. Differentiation of dextran-producing *Leuconostoc* strains from fermented rice cake (*puto*) using pulsed-field gel electrophoresis. Int. J. Food Microbiol. 26: 345 - 352.

Kelly, W. J., Huang, C. M. and R. V. Asmundson. 1993. Comparison of *Leuconostoc oenos* strains by pulsed-field gel electrophoresis. Appl. Environ. Microbiol. 59: 3969-3972.

Kempton, A. G. and S. R. Bobier. 1970. Bacterial growth in refrigerated, vacuum-packed luncheon meats. Can. J. Microbiol. 16: 287 - 297.

Kemp, D. J., Corcoran, L. M., Coppel, R. L., Stahl, H. D., Bianco, A. E., Brown, G. V. and R. F. Anders. 1985. Size variation in chromosomes from independent cultured isolates of *Plasmodium falciparum*. Nature, 315: 347-350.

Kitchell, A. G. and B. G. Shaw. 1975. Lactic acid bacteria in fresh and cured meat. *in* Lactic Acid Bacteria in Beverages and Food. (Carr, J. G., Cutting, J. G. and Whiting, C. V. eds.) pp. 209 - 220. Academic Press, London.

Klaenhammer, T. R. 1988. Bacteriocins of lactic acid bacteria. Biochemie, 70: 337 - 349.

Klaenhammer, T. R. 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. 12: 39 - 86.

Klein, G., Dicks, L. M. T, Pack, A., Hack, B., Zimmermann, K., Dellaglio, F. and G. Reuter. 1996. Emended descriptions of *Lactobacillus sake* (Katagiri, Katahara and Fukami) and *Lactobacillus curvatus* (Abo-Elnaga and Kandler): Numerical classification revealed by protein fingerprinting and identification based on biochemical patterns and DNA-DNA hybridizations. Int. J. Syst. Bacteriol. 46: 367 - 376.

Korkeala, H. J., Alanko, T. and T. Tiusanen. 1992. Effect of sodium nitrite and sodium chloride on growth of lactic acid bacteria. Acta Vet. Scand. 33: 27 - 32.

Korkeala, H. J., Alanko, T., Makela, P. and S. Lindroth. 1989. Shelf-life of vacuum-packed cooked ring sausages at different chill temperatures. Int. J. Food Microbiol. 9: 237 - 247.

Korkeala, H. J. Makela, P. and H. Suominen. 1990. Growth temperatures of ropy slime-producing lactic acid bacteria. J. Food. Prot. 53: 793 - 794.

Korkeala, H. J., Alanko, T. Makela, P. and S. Lindroth. 1990. Lactic acid and pH as quick indicators of spoilage in vacuum-packed cooked ring sausages. Int. J. Food Microbiol. 10: 245 - 254.

Korkeala, H. J. and K. J. Bjorkroth. 1997. Microbiological spoilage and contamination of vacuum-packaged cooked sausages. J. Food Prot. 60: 724 - 731.

Korkeala H. J. and S. Lindroth. 1987. Differences in microbial growth in the surface layer and at the center of vacuum-packed cooked ring sausages. Int. J. Food Microbiol. 4: 105 - 110.

Korkeala, H. J., Lindroth, S., Ahvenainen, R. and T. Alanko. 1987. Interrelationship between different parameters in the spoilage of vacuum-packed cooked ring sausages. Int. J. Food Microbiol. 5: 311 - 321.

Korkeala, H. J., Suortti, T. and P. Makela. 1988. Ropy slime formation in vacuum-packed cooked meat products caused by homofermentative lactobacilli and a *Leuconostoc* species. Int. J. Food Microbiol. 7: 339-347.

Levene, S. D. 1992. Theories of pulsed-field gel electrophoresis. *in* Pulsed-Field Gel Electrophoresis: Protocols, Methods and Theories. (Burmeister, M. and Ulanovsky, L., eds.) pp. 347-365. Humana Press, Totowa, NJ.

Makela, P. and H. J. Korkeala. 1987. Lactobacilli contamination of a cooked ring sausage processing plant. Int. J. Food Microbiol. 5: 323 - 330.

Makela, P. and H. J. Korkeala. 1992. The ability of the ropy slime-producing bacteria to form ropy colonies on different culture media and different incubation temperatures and atmospheres. Int. J. Food Microbiol. 16: 161-166.

Makela, P., Korkeala, H. J. and J. Laine. 1992. Survival of ropy slime-producing lactic acid bacteria in heat processes used in the meat industry. Meat Sci. 31: 463 - 471.

Makela, P. Schillinger, U., Korkeala, H. and W. H. Holzapfel. 1992. Classification of ropy slime-producing lactic acid bacteria based on DNA-DNA homology, and identification of *Lactobacillus sake* and *Leuconostoc amelibiosum* as dominant spoilage organisms in meat products. Int. J. Food Microbiol. 16: 167 - 172.

McDonald, L. C. McFeeters, R. F., Daeschel, M. A. and H. P. Fleming. 1987. A differential medium for the enumeration of homofermentative and heterofermentative lactic acid bacteria. Appl. Environ. Microbiol. 53: 1382 - 1384.

Meng, J., Zhao, S., Zhao, T. and M. P. Doyle. 1995. Molecular characterization of *Escherichia coli* O157:H7 isolates by pulsed-field gel electrophoresis and plasmid DNA analysis. J. Med. Microbiol. 42: 258-263.

Mol, J. H. H., Hietbrink, J. E. A., Mollen, H.W.M. and J. van Tinteren. 1971. Observations on the microflora of vacuum packed sliced cooked meat products. J. Appl. Bacteriol. 34: 377 - 397.

Muriana, P. M. 1996. Bacteriocins for control of *Listeria* spp. in food. J. Food. Prot. 1996 supplement: 54 - 63.

Nielsen, H. -J. S. 1983. Influence of temperature and gas permeability on the storage life and microbiology of vacuum-packed meat. J. Appl. Bacteriol. 47: 433 - 441.

Nissen, H., Sørheim, O. and R. Dainty. 1996. Effects of vacuum, modified atmosphere and storage temperature on the microbial flora of packaged beef. Food Microbiol. 13: 183-191.

Niven, C. F. Jr., Smiley, K. L. and J. M. Sherman. 1942. The hydrolysis of arginine by streptococci. J. Bacteriol. 43: 651 - 660.

Papadopoulos, L. S., Miller, R. K., Acuff, G.R., Vanderzant, C. and H. R. Cross. 1991. Effect of sodium lactate on microbial and chemical composition of cooked beef during storage. J. Food Sci. 56: 341 - 347.

Pearson, A. M. and F. W. Tauber. 1984. Curing. *in* Processed Meat. pp. 46-67. AVI Publishing Company, Inc., Westport, CT.

Reid, T. F. 1969. Lactic acid and lactates in food products. Food Manufacture, 44: 54 - 55.

Reuter, G. 1970. Lactobacilli and closely related microorganisms in meat and meat products. 2. Characterization of isolated lactobacilli strains. Fleischwirtschaft, 50: 954 - 962.

Roberts, T. A., Gibson, A. M. and A. Robinson. 1981. Factors controlling the growth of Clostridium botulinum type A and B in pasteurized cured meats. J. Food Technol. 16: 239-266.

Schillinger, U. and F. -K. Lucke. 1987. Identification of lactobacilli from meat and meat products. Food Microbiol. 4: 199 - 208.

Schillinger, U. and F. -K. Lucke. 1989. Antibacterial activity of *Lactobacillus sake* isolated from meat. Appl. Environ. Microbiol. 55: 204 - 217.

Sharpe, M. E., Fryer, T. F. and D. G. Smith. 1996. Identification of the lactic acid bacteria *in* Identification Methods for Microbiologists. (Gibbs, B. M. and Skinner, F. A. eds.) Part A. pp. 65 - 79. Academic Press, New York, NY.

Shaw, B. G. and C. D. Harding. 1984. A numerical taxonomic study of lactic acid bacteria from vacuum-packed beef, pork, lamb and bacon. J. Appl. Bacteriol. 56: 25 - 40.

Sip, A., Grajek, W. and P. Boyaval. 1998. Enhancement of bacteriocin production by *Carnobacterium divergens* AS7 in the presence of a bacteriocin-sensitive strain *Carnobacterium piscicola*. Int. J. Food Microbiol. 42:63-69.

Smith, C. L. and C. R. Cantor. 1987. Purification, specific fragmentation, and separation of large DNA molecules. Methods Enzymol. 155: 449-467.

Sneath, P. H. A. and D. Jones. 1976. *Brochothrix*, a new genus tentatively placed in the family *Lactobacillaceae*. Int. J.Syst. Bacteriol. 26: 102 - 104.

Stiles, M. E. and W. H. Holzepfel. 1997. Lactic acid bacteria of foods and their current taxonomy. Int. J. Food Microbiol. 36: 1 - 29.

Tanskanen, E. I., Tulloch, D. L., Hillier, A. J. and B. E. Davidson. 1990. Pulsed-field gel electrophoresis of *Sma*I digests of lactococcal genomic DNA, a novel method of strain identification. Appl. Environ. Microbiol. 56: 3105- 3111.

Tenover, F. C., Arbeit, R. D., Goering, R. V., Mickelsen, P. A., Murray, B. E., Persing, D. H. and B. Swaminathan. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. 33: 2233-2239.

Townsend, W. E. and D. G. Olson. 1987. Cured meats and cured meat products processing. *in* The Science of Meat and Meat Products. (Price, J. F. and Schweigert, B. S., eds.) pp. 431-456. Food & Nutrition Press Inc., Westport, CT.

User manual. 1995. Gene Navigator system. Pharmacia Biotech AB, Uppsala, Sweden. 81pp.

USDA, APHIS. 1975. Nitrates, nitrites and ascorbates (or isoascorbates) in bacon. Federal Register 40, 52614.

Von Holy, A., Cloete, T. E. and W. H. Holzapfel. 1991. Quantification and characterization of microbial populations associated with spoiled, vacuum-packed Vienna sausages. Food Microbiol. 8: 95 - 104.

Watts, B. M. 1954. Oxidative rancidity and discoloration in meat. Adv. Food Res. 5: 1-9.

Yang, R. and B. Ray. 1994. Prevalence and biological control of bacteriocin-producing psychrotrophic leuconostocs associated with spoilage of vacuum-packed processed meats. J. Food Prot. 57: 204 - 217.

Zuniga, M., Pardo, I. and S. Ferrer. 1993. An improved medium for distingushing between homofermentative and heterofermentative lactic acid bacteria. Int. J. Food Microbiol. 18: 37 - 42.

Appendices

Appendix 1. Chemicals

Chemicals	Manufacturers
Trypticase peptone	Becton Dickinson and Co., Cockeysville, MD
Yeast extract	Difco Laboratories, Detroit, MI
Protease peptone No.3	Difco Laboratories, Detroit, MI
Beef extract	Difco Laboratories, Detroit, MI
Agar granulated	Becton Dickinson and Co., Cockeysville, MD
Tween 80 (polysorbate 80 USP)	Fisher Scientific Inc., Nepean, ON
Fructose	Mallinckrodt Specialty Chemicals Co., Paris, KT
D-(+)-glucose	Sigma Chemical Co., St. Louis, MO
D-(+)-trehalose	Sigma Chemical Co., St. Louis, MO
Melibiose	Sigma Chemical Co., St. Louis, MO
Maltose	Difco Laboratories, Detroit, MI
L-(+)-arabinose	Sigma Chemical Co., St. Louis, MO
D-amygdalin	Sigma Chemical Co., St. Louis, MO
Potassium dihydrogen phosphate (KH_2PO_4)	Mallinckrodt Specialty Chemicals Co., Paris, KT

Appendix 1. *(Continued)*

Chemicals	Manufacturers
Dipotassium hydrogen phosphate (K_2HPO_4)	Mallinckrodt Specialty Chemicals Co., Paris, KT
Disodium hydrogen phosphate (Na_2HPO_4)	Mallinckrodt Specialty Chemicals Co., Paris, KT
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	Mallinckrodt Specialty Chemicals Co., Paris, KT
Manganese sulfate ($MnSO4 \cdot H_2O$)	Mallinckrodt Specialty Chemicals Co., Paris, KT
Hydrochloric acid (HCl)	Fisher Scientific Inc., Nepean, ON
Sodium hydroxide (NaOH)	Mallinckrodt Specialty Chemicals Co., Paris, KT
Potassium hydroxide (KOH)	Mallinckrodt Specialty Chemicals Co., Paris, KT
Hydrogen peroxide (H_2O_2)	Fisher Scientific Inc., Nepean, ON
Barium chloride ($BaCl_2$)	Mallinckrodt Specialty Chemicals Co., Paris, KT
Sulfuric acid (H_2SO_4)	Fisher Scientific Inc., Nepean, ON
Sodium chloride (NaCl)	Sigma Chemical Co., St. Louis, MO
Sodium nitrite ($NaNO_2$)	J.T.Baker Chemical Co., Phillipsburg, NJ

Appendix 1. *(Continued)*

Chemicals	Manufacturers
Potassium iodide (KI)	Fisher Scientific Inc., Nepean, ON
Mercuric iodide (HgI_2)	Fisher Scientific Inc., Nepean, ON
Ammonium citrate	Anachemia, Rouses Point, NY
Sodium acetate	Mallinckrodt Specialty Chemicals Co., Paris, KT
Sodium citrate	Anachemia, Rouses Point, NY
Calcium pantothenate	Sigma Chemical Co., St. Louis, MO
Polymyxin B sulfate	Sigma Chemical Co., St. Louis, MO
L-cysteine hydrochloride	Sigma Chemical Co., St. Louis, MO
L-arginine hydrochloride	Sigma Chemical Co., St. Louis, MO
Bromocresol green	Sigma Chemical Co., St. Louis, MO
Bromocresol purple	Sigma Chemical Co., St. Louis, MO
Phenol red	Difco Laboratories, Detroit, MI
Fast green FCF	Anachemia, Rouses Point, NY
Mineral oil	Sigma Chemical Co., St. Louis, MO
Disodium EDTA	Fisher Scientific Inc., Nepean, ON

Appendix 1. *(Continued)*

Chemicals	Manufacturers
TrisHCl	Sigma Chemical Co., St. Louis, MO
Sarkosyl (N-lauroylsarcosine)	Sigma Chemical Co., St. Louis, MO
Sarcosine	Sigma Chemical Co., St. Louis, MO
Mutanolysin	Sigma Chemical Co., St. Louis, MO
RNase (ribonuclease A)	Sigma Chemical Co., St. Louis, MO
Proteinase K	Sigma Chemical Co., St. Louis, MO
Catalase (from bovine liver)	Sigma Chemical Co., St. Louis, MO
Trypsin	Sigma Chemical Co., St. Louis, MO
Protease	Sigma Chemical Co., St. Louis, MO
Agarose (for PFGE running gel)	Sigma Chemical Co., St. Louis, MO
Low melting point agarose	Sigma Chemical Co., St. Louis, MO
Phenylmethylsulfonyl fluoride (PMSF)	Sigma Chemical Co., St. Louis, MO
Acetylated bovine serum albumin	Promega Corporation, Madison, WI
Restriction endonuclease *Sma*I	Promega Corporation, Madison, WI
Restriction endonuclease *Avr*II	Promega Corporation, Madison, WI

Appendix 1. *(Continued)*

Chemicals	Manufacturers
Ethidium bromide	Sigma Chemical Co., St. Louis, MO
Pulse marker (DNA size standards, 0.1-200 kb)	Sigma Chemical Co., St. Louis, MO
Morpholinoethane sulphonic acid (MES)	Fisher Scientific Inc., Nepean, ON

Appendix 2. Media and reagents

Media/reagents	Manufacturers/references
MRS broth	Difco Laboratories, Detroit, MI
APT broth	Becton Dickinson and Co., Cockeysville, MD
M5 agar	Zuniga et al., 1995
MRSD agar	Holley and Millard, 1993
CO_2 production test broth	Schillinger and Lucke, 1987
Arginine hydrolysis test broth	Schillinger and Lucke, 1987
Nessler's reagent	Harrigan and McCance, 1976
Sugar fermentation test broth	Schillinger and Lucke, 1987
McFarland standards	Finegold and Martin, 1982
TrisHCl-EDTA (TE) buffer (pH 7.8)	Sigma Chemical Co., St. Louis, MO
TrisHCl-Borate-EDTA (TBE) buffer	Sigma Chemical Co., St. Louis, MO
EDTA-Sarcosine (ES) buffer (pH 9.2)	Smith and Cantor, 1987
EDTA-Proteinase K-Sarcosine(ESP) buffer (pH 9.2)	Smith and Cantor, 1987
Restriction endonuclease buffer	Promega Corporation, Madison, WI

Appendix 2. *(Continued)*

Media/reagents	Manufacturers/references
Modified MRS (MMRS) broth	Formula and preparation procedures are specified in the Methods part.

Appendix 3. Supplies and instruments

Supplies/instruments	Manufacturers
Iso-grid® hydrophobic grid membrane filters (HGMF), filtration towers and clamps	QA Labs Ltd., Toronto, ON
Petri dishes	Fisher Scientific Inc., Nepean, ON
Seward medical stomacher bags and Stomacher lab-blender, model 400	Seward Laboratory, London, UK
Accumet® pH meter 910	Fisher Scientific Inc., Nepean, ON
Stainless steel core device (306 stainless steel pipe with beveled edge, 10 cm in diameter)	manufactured in workshop, University of Manitoba
0.2 um, 25 mm disposable Nalgene syringe filters	Fisher Scientific Inc., Nepean, ON
5 cc disposable B-D syringes and needles	Fisher Scientific Inc., Nepean, ON
0.1~10, 1~100, 100~1,000 Eppendorf Reference pipetters	Fisher Scientific Inc., Nepean, ON
Vortex Genie-2 mixer	Fisher Scientific Inc., Nepean, ON
BBL GasPak® CO_2 atmosphere anaerobic system (including jars, disposable GasPak Plus® envelops with palladium catalyst and disposable anaerobic indicators	Becton Dickinson and Co., Cockeysville, MD
Quebec® dark-field colony counter	American Optical Scientific Instrument Division, Buffalo, NY
Microscope slides and cover glass	Fisher Scientific Inc., Nepean, ON

Appendix 3. *(Continued)*

Supplies/instruments	Manufacturers
API 50 CHL carbohydrate metabolism system	BioMérieux SA, Marcy-l'Etoile, France
Deli #1 high O_2 barrier pouches	Winpak Inc., Winnipeg, MB
Bizerba GM 2002 vacuum packager	Bizerba Inc., Mississauga, ON
Sorvall superspeed RC2-B automatic refrigerated centrifuge	Ivan Sorvall Inc., Norwalk, CT
Zeiss universal research microscope	Carl Zeiss Inc., Oberkochen, Germany
96 well Falcon® 3072 Microtest III tissue culture plates	Becton Dickinson and Co., Lincoin Park, NJ
Gene Navigator® pulsed-field gel electrophoresis system (including the electrophoresis unit, hexagonal electrode, GN controller, ESP 600 power supply and other accessories such as molds, combs, gel supporting tray and staining tray)	Pharmacia Biotech AB, Uppsala, Sweden
Haake G thermostatic circulator	Gebr. Haake GmbH, Karlsruhe, Germany
Ultraspec 2000 UV/visible spectrophotometer	Pharmacia Biotech AB, Uppsala, Sweden
Disposable 4.5 ml plastic cuvettes	Fisher Scientific Inc., Nepean, ON
Parafilm "M"® laboratory film	American National Can Inc., Chicago, IL

Appendix 3. *(Continued)*

Supplies/instruments	Manufacturers
Gel Doc 1000 video gel documentation system	Bio-Rad Laboratories Ltd., Richmond, CA
controlled environment incubator shaker	Lab-Line Instruments, Inc., Melrose Park, IL
Polaroid 667 film	Polaroid Canada Inc., Etobicoke, ON
Polaroid MP4 land camera	Polaroid Canada Inc., Etobicoke, ON

Appendix 4. Standard procedure for LAB strain maintenance. (Adopted from G. E. Millard, Center for Food & Animal Research, Agriculture and Agri-Food Canada, Ottawa)

1. Grow culture 24-48 h using APT (MRS) broth aerobically at 25°C.
2. Streak for purity. Examine for morphology, Gram reaction (3% KOH), catalase (3% H_2O_2). Re-isolate back to APT (MRS) broth for a second period of growth.
3. Centrifuge the second broth culture. Decant the supernatant and resuspend the pellet in 1.5 ml fresh broth.
4. Add 0.75 ml filter-sterilized glycerol (50% v/v) and mix thoroughly.
5. Add 0.3 ml of the final suspension to each of 6 labeled cryogenic vials and freeze at -80°C.
6. Check resuscitation of one vial after more than 30 days storage and if successful, store remaining vials in coded containers at -80°C indefinitely.

Appendix 5. Carbohydrate utilization by the spoilage LAB isolated from meat samples using the API 50 CHL System.[1]

	Isolate	R1	C1	CB1	CB2	CB3	CB4	CB5	CB6	CB7
0	Control									
1	Glycerol									
2	Erythritol									
3	D-Arabinose									
4	L-Arabinose		+		+				+	
5	Ribose	+	+	+		+	+	+		+
6	D-Xylose		+		+				+	
7	L-Xylose									
8	Adonitol									
9	β-Methyl-xyloside									
10	Galactose	+	+		+	+	+	+	+	+
11	D-Glucose	+	g	g	g	+	+	+	g	+
12	D-Fructose	+	g	g	g	+	+	+	g	+
13	D-Mannose	+	+	+	g	+	+	+	g	+
14	L-Sorbose									
15	Rhammose									
16	Dulcitol									
17	Inositol									
18	Mannitol		+		+				+	
19	Sorbitol									
20	α-Methyl-D-mannoside									
21	α-Methyl-D-glucoside		+	+	+	+		+	+	+

Appendix 5. (*Continued*)

	Isolate	R1	C1	CB1	CB2	CB3	CB4	CB5	CB6	CB7
22	N-Acetyl-glucosamine	+	+	+	g	+	+	+	g	+
23	Amygdalin									
24	Arbutin		+		+				+	
25	Esculin		+		+				+	
26	Salicin		+		+	+		+	+	+
27	Cellobiose									
28	Maltose	+	g	+	g		+	+	g	
29	Lactose	+	+		+	+			+	+
30	Melibiose		g		g	+	+	+	g	+
31	Sucrose		g	g	g	+	+	+	g	+
32	Trehalose		+		+	+	+	+	+	+
33	Inulin									
34	Melezitose									
35	D-Raffinose		+		+				+	
36	Starch									
37	Glycogen									
38	Xylitol									
39	β Gentiobiose									
40	D-Turanose		g	+	+		+		+	
41	D-Lyxose									
42	D-Tagatose									
43	D-Fucose									
44	L-Fucose									
45	D-Arabitol									

Appendix 5. (*Continued*)

	Isolate	R1	C1	CB1	CB2	CB3	CB4	CB5	CB6	CB7
46	L-Arabitol									
47	Gluconate		g	+		g	g	g		g
48	2-Keto-gluconate		g							
49	5-Keto-gluconate		+							

---

[1] Symbols: "+" - positive result (the color of the test medium changed from blue to yellow). "g" - positive result accompanying gas production. Space - negative result.

Appendix 6. Growth ability of a number of spoilage LAB strains in standard MMRS broth.

Leuconostoc mesenteroides		*L. curvatus*		*L. sake*	
Strain	LogCFU/ml	Strain	LogCFU/ml	Strain	LogCFU/ml
No. 1	7.40 ± 0.43^{2a}	No. 3	7.25 ± 0.68^{a}	No. 6	7.80 ± 0.61^{a}
No.11	7.14 ± 0.19^{a}	No. 5	7.55 ± 0.44^{a}	No. 9	8.21 ± 0.32^{a}
No.13	7.50 ± 0.33^{a}	No.10	7.32 ± 0.55^{a}	No.14	7.67 ± 0.40^{ab}
No.33	7.02 ● 0.21^{a}	No.15	7.53 ± 0.42^{a}	No.34	6.91 ± 0.27^{b}
		No.23	7.36 ± 0.44^{a}		
		No.35	7.92 ± 0.22^{a}		

[1] Numbers of bacteria were determined after incubation at 6°C anaerobically for 2 weeks (see Chapter 3).

[2] Mean viable cell concentrations of 3 replicates ± standard deviation.

[a,b] Means within the same column with different superscript are significantly different ($p < 0.05$).

Appendix 7. Growth of LAB in MMRS broth and the final pH of the broth with different concentrations of NaCl.[1]

Strain[2]	LAB (logCFU/ml) at different NaCl concentrations (%)		
	2.5%	4%	5.5%
# 1	7.44 ± 0.10[3]^{a} (5.84) [4]	5.94 ± 0.23^{b} (5.93)	5.62 ± 0.26^{b} (5.92)
#11	7.07 ± 0.22^{a} (5.88)	6.22 ± 0.14^{b} (5.93)	6.15 ± 0.08^{b} (5.92)
# 6	7.37 ± 0.06^{a} (5.77)	5.58 ± 0.64^{b} (5.92)	5.67 ± 0.35^{b} (5.93)
# 9	8.33 ± 0.25^{a} (5.05)	7.13 ± 0.30^{b} (5.86)	7.14 ± 0.45^{b} (5.85)
#10	6.82 ± 0.21^{a} (5.87)	6.64 ± 0.09^{a} (5.94)	6.70 ± 0.34^{a} (5.91)
#15	7.58 ± 0.27^{a} (5.70)	7.63 ± 0.17^{a} (5.92)	7.36 ± 0.16^{a} (5.90)

[1] number of bacteria were determined after incubation at 6°C anaerobically for 2 weeks (see Chapter 3).

[2] strain identities are listed in appendix 6.

[3] mean viable cell concentrations of 3 replicates ± standard deviation.

[4] mean final pH values of 3 replicates (all standard deviations < 0.05)

^{a,b} means within the same row with different superscript are significantly different ($P<0.05$).

Appendix 8. Growth of LAB in MMRS broth and the final pH of the broth with different concentrations of $NaNO_2$[1]

Strain[2]	LAB (logCFU/ml) at different $NaNO_2$ levels (ppm)		
	100	150	200
# 1	7.44 ± 0.10[3a] (5.84)[4]	7.07 ± 0.19[a] (5.95)	7.13 ± 0.09[a] (5.91)
#11	7.07 ± 0.22[a] (5.88)	7.10 ± 0.14[a] (5.94)	7.00 ± 0.49[a] (5.91)
# 6	7.37 ± 0.06[a] (5.77)	7.38 ± 0.13[a] (5.81)	7.60 ± 0.46[a] (5.88)
# 9	8.33 ± 0.25[a] (5.05)	8.06 ± 0.07[a] (5.09)	8.37 ± 0.16[a] (5.37)
#10	6.82 ± 0.21[a] (5.87)	7.16 ± 0.23[a] (5.91)	6.96 ± 0.45[a] (5.89)
#15	7.58 ± 0.27[a] (5.70)	8.06 ± 0.28[a] (5.78)	7.71 ± 0.18[a] (5.82)

[1] number of bacteria were determined after incubation at 6°C anaerobically for 2 weeks (see Chapter 3).

[2] strain identities are listed in appendix 6.

[3] mean viable cell concentrations of 3 replicates ± standard deviation.

[4] mean final pH values of 3 replicates (all standard deviations < 0.05)

[a] means within the same row with different superscript are significantly different (P<0.05).

Appendix 9. Growth of LAB in MMRS broth and final pH of the broth with different initial pH.[1]

Strain[2]	LAB (logCFU/ml) at different initial pH		
	5.5	6.0	6.5
# 1	6.46 ± 0.08[3a] (5.41)[4]	7.44 ± 0.10[b] (5.84)	7.97 ± 0.25[c] (6.31)
#11	6.56 ± 0.05[a] (5.43)	7.07 ± 0.22[b] (5.88)	7.26 ± 0.08[b] (6.35)
# 6	6.40 ± 0.50[a] (5.40)	7.37 ± 0.06[b] (5.77)	6.46 ± 0.08[a] (6.21)
# 9	7.35 ± 0.31[a] (5.13)	8.33 ± 0.25[b] (5.05)	7.48 ± 0.10[a] (5.34)
#10	7.15 ± 0.47[a] (5.41)	6.82 ± 0.21[a] (5.87)	6.82 ± 0.05[a] (6.28)
#15	7.49 ± 0.12[a] (5.24)	7.58 ± 0.27[a] (5.70)	7.34 ± 0.13[a] (6.12)

[1] number of bacteria were determined after incubation at 6°C anaerobically for 2 weeks (see Chapter 3).

[2] strain identities are listed in appendix 6.

[3] mean viable cell concentrations of 3 replicates ± standard deviation.

[4] mean final pH values of 3 replicates (all standard deviations < 0.05)

[a,b,c] means within the same row with different superscript are significantly different ($P<0.05$).

Appendix 10. Growth of LAB in MMRS broth and the final pH of the broth at different incubation temperature.[1]

Strain[2]	LAB (logCFU/ml) at different temperature (°C)		
	2°C	6°C	12°C
# 1	8.12 ± 0.38[3]^{a} (5.81)[4]	7.44 ± 0.10^{b} (5.84)	7.38 ± 0.28^{b} (6.31)
#11	7.98 ± 0.19^{a} (5.67)	7.07 ± 0.22^{a} (5.88)	7.40 ± 1.17^{a} (5.53)
# 6	8.20 ± 0.12^{a} (5.06)	7.37 ± 0.06^{b} (5.77)	7.47 ± 0.09^{b} (5.18)
# 9	8.15 ± 0.14^{a} (4.48)	8.33 ± 0.25^{a} (5.05)	7.18 ± 0.43^{b} (4.45)
#10	7.24 ± 0.09^{b} (5.71)	6.82 ± 0.21^{a} (5.87)	8.35 ± 0.23^{c} (5.34)
#15	7.77 ± 0.36^{a} (5.55)	7.58 ± 0.27^{a} (5.70)	8.75 ± 0.14^{b} (5.23)

[1] number of bacteria were determined after 5 weeks for those incubated at 2°C, 2 weeks for those incubated at 6°C and one week for those incubated at 12°C (see Chapter 3).

[2] strain identities are listed in appendix 6.

[3] mean viable cell concentrations of 3 replicates ±standard deviation.

[4] mean final pH values of 3 replicates (all standard deviations < 0.05)

[a,b,c]means within the same row with different superscript are significantly different (P<0.05).

Appendix 11. Effect of initial pH and incubation temperature on the growth of *Lc. mesenteroides* strain No. 1 and *L. curvatus* strain No. 10 in mixed inocula at an initial ratio of 10^4:10^4 (CFU/ml).[1]

Temperature	pH	Lc (logCFU/ml)	Lbc (logCFU/ml)	log(Ratio)[2]
12°C	5.5	7.07 ± 0.24[3]	8.48 ± 0.26	-1.41
12°C	6.0	6.77 ± 0.29	8.93 ± 0.04	-2.16
12°C	6.5	7.04 ± 0.08	8.81 ± 0.19	-1.77
6°C	5.5	4.67 ± 0.58	4.97 ± 0.35	-1.30
6°C	6.0	8.23 ± 0.25	5.56 ± 0.25	2.67
6°C	6.5	7.31 ± 0.42	5.18 ± 0.15	2.13
2°C	5.5	2.58 ± 1.15	3.36 ± 1.22	-0.78
2°C	6.0	<4.00	6.78 ± 2.00	<-2.78
2°C	6.5	5.50 ± 0.24	5.50 ± 1.13	0

[1] symbols: Lc: numbers of *Leuconostoc mesenteroides* (logCFU/ml); Lbc: numbers of *Lactobacillus curvatus* (logCFU/ml).

[2] log (Ratio) = Lc - Lbc.

[3] mean viable cell concentrations of 3 replicates ± standard deviation.

Appendix 12. Effect of initial pH and incubation temperature on the growth of *Lc. mesenteroides* strain No.1 and *L. sake* strain No.9 in mixed inocula at an initial ratio of 10^4:10^4 (CFU/ml).[1]

Temperature	pH	Lc (logCFU/ml)	Lbs (logCFU/ml)	log(Ratio) [2]
12°C	5.5	5.70 ± 0.82[3]	5.41 ± 1.14	0.29
12°C	6.0	7.44 ± 0.35	7.67 ± 0.17	-0.23
12°C	6.5	8.00 ± 0.06	7.69 ± 0.12	0.31
6°C	5.5	3.99 ± 1.19	4.11 ± 1.41	-0.12
6°C	6.0	7.55 ± 0.23	7.43 ± 0.20	0.12
6°C	6.5	7.03 ± 0.23	7.18 ± 0.14	-0.15
2°C	5.5	3.00 ± 1.73	4.11 ± 0.62	-1.11
2°C	6.0	<4.00	7.61 ± 0.32	<-3.61
2°C	6.5	4.01 ± 0.02	6.90 ± 1.25	-2.89

[1] symbols: Lc: numbers of *Leuconostoc mesenteroides* (logCFU/ml); Lbs: numbers of *Lactobacillus sake* (logCFU/ml).

[2] log (Ratio) = Lc - Lbs.

[3] mean viable cell concentrations of 3 replicates ± standard deviation.

Appendix 13. Effect of initial pH and incubation temperature on the growth of *Lc. mesenteroides* strain No.1 and *L. curvatus* strain No.10 in mixed inocula at an initial ratio of 10^7:10^4 (CFU/ml).[1]

Temperature	pH	Lc (logCFU/ml)	Lbc (logCFU/ml)	log(Ratio)[2]
12°C	5.5	7.80 ± 0.05[3]	<6.00	>1.80
12°C	6.0	7.86 ± 0.26	<6.00	>1.86
12°C	6.5	7.39 ± 0.20	<6.00	>1.39
6°C	5.5	7.57 ± 0.40	6.04 ± 0.06	1.53
6°C	6.0	7.79 ± 0.06	<6.00	>1.79
6°C	6.5	7.73 ± 0.13	<6.00	>1.73
2°C	5.5	5.00 ± 0.67	4.97 ± 0.66	0.03
2°C	6.0	6.38 ± 1.29	5.72 ± 0.97	0.66
2°C	6.5	5.59 ± 1.14	4.48 ± 0.40	1.11

[1] symbols: Lc: numbers of *Leuconostoc mesenteroides* (logCFU/ml); Lbc: numbers of *Lactobacillus curvatus* (logCFU/ml).

[2] log (Ratio) = Lc - Lbc.

[3] mean viable cell concentrations of 3 replicates ● standard deviation.

Appendix 14. Effect of initial pH and incubation temperature on the growth of *Lc. mesenteroides* strain No.1 and *L. sake* strain No.9 in mixed inocula at an initial ratio of 10^7:10^4 (CFU/ml).[1]

Temperature	pH	Lc (logCFU/ml)	Lbs (logCFU/ml)	log(Ratio)[2]
12°C	5.5	8.20 ● 0.16[3]	<6.00	>2.20
12°C	6.0	8.24 ± 0.28	<6.00	>2.24
12°C	6.5	8.12 ± 0.54	6.16 ± 0.27	1.96
6°C	5.5	8.13 ± 0.20	6.31 ± 0.24	1.82
6°C	6.0	7.88 ± 0.23	6.16 ± 0.27	1.72
6°C	6.5	7.16 ± 0.22	6.18 ± 0.38	0.98
2°C	5.5	5.88 ± 0.12	4.89 ± 0.61	0.99
2°C	6.0	6.23 ± 1.06	5.67 ± 1.29	0.56
2°C	6.5	5.85 ± 0.54	5.59 ± 0.46	0.26

[1] symbols: Lc: numbers of *Leuconostoc mesenteroides* (logCFU/ml); Lbs: numbers of *Lactobacillus sake* (logCFU/ml).

[2] log (Ratio) = Lc - Lbs.

[3] mean viable cell concentrations of 3 replicates ● standard deviation.

Appendix 15. Effect of initial pH and incubation temperature on the growth of *Lc. mesenteroides* strain No. 1 and *L. curvatus* strain No. 10 in mixed inocula at an initial ratio of 10^4:10^7 (CFU/ml).[1]

Temperature	pH	Lc (logCFU/ml)	Lbc (logCFU/ml)	log(Ratio) [2]
12°C	5.5	<6.00	8.65 ± 0.31	<-2.65
12°C	6.0	<6.00	8.83 ± 0.14	<-2.83
12°C	6.5	6.00 ± 0.01	7.99 ± 0.18	-1.99
6°C	5.5	6.65 ± 0.62	8.66 ± 0.28	-2.01
6°C	6.0	6.32 ± 0.23	8.83 ± 0.09	-2.51
6°C	6.5	6.40 ± 0.34	8.77 ± 0.14	-2.37
2°C	5.5	<6.00	8.13 ± 0.24	<-2.13
2°C	6.0	6.11 ± 0.16	8.12 ± 0.40	-2.01
2°C	6.5	6.10 ± 0.17	8.11 ± 0.46	-2.01

[1] symbols: Lc: numbers of *Leuconostoc mesenteroides* (logCFU/ml); Lbc: numbers of *Lactobacillus curvatus* (logCFU/ml).

[2] log (Ratio) = Lc - Lbc.

[3] mean viable cell concentrations of 3 replicates ± standard deviation.

Appendix 16. Effect of initial pH and incubation temperature on the growth of *Lc. mesenteroides* strain No.1 and *L. sake* strain No.9 in mixed inocula at an initial ratio of 10^4:10^7 (CFU/ml).[1]

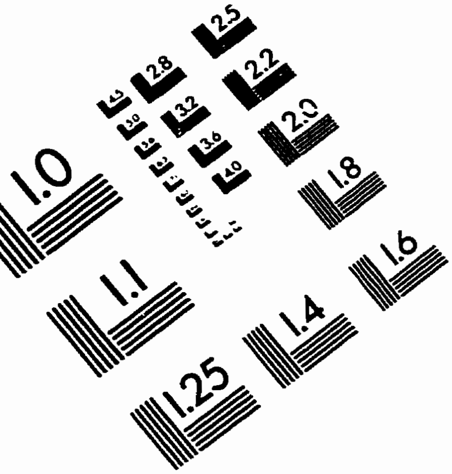
Temperature	pH	Lc (logCFU/ml)	Lbs (logCFU/ml)	log(Ratio)[2]
12°C	5.5	<6.00	8.16 ± 0.29	<-2.16
12°C	6.0	<6.00	8.21 ± 0.15	<-2.21
12°C	6.5	<6.00	8.26 ± 0.47	<-2.26
6°C	5.5	<6.00	7.91 ± 0.19	<-1.91
6°C	6.0	<6.00	8.03 ± 0.33	<-2.03
6°C	6.5	<6.00	7.75 ± 0.32	<-1.75
2°C	5.5	<6.00	7.62 ± 0.22	<-1.62
2°C	6.0	<6.00	7.55 ± 0.28	<-1.55
2°C	6.5	<6.00	7.36 ± 0.31	<-1.36

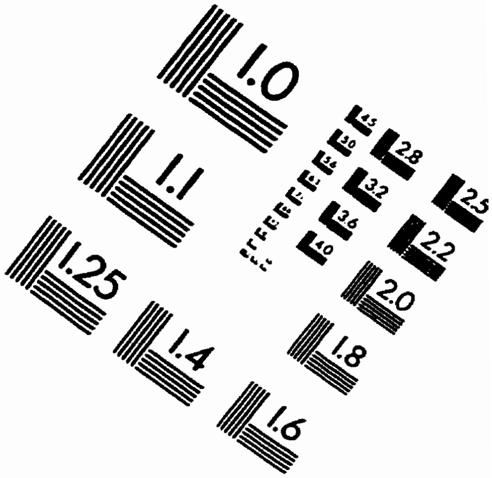
[1] symbols: Lc: numbers of *Leuconostoc mesenteroides* (logCFU/ml); Lbs: numbers of *Lactobacillus sake* (logCFU/ml).

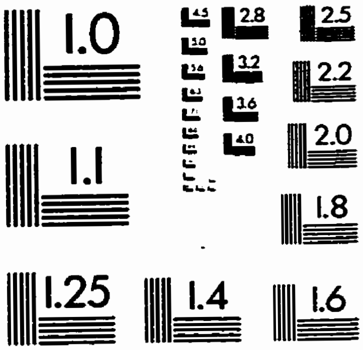
[2] log (Ratio) = Lc - Lbs.

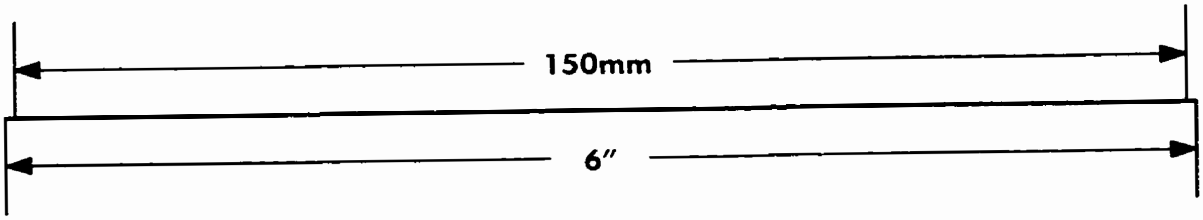
[3] mean viable cell concentrations of 3 replicates ● standard deviation.

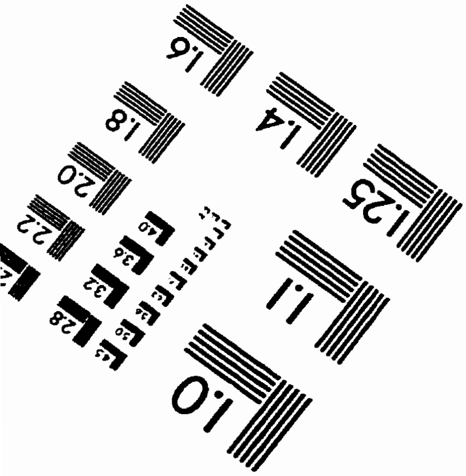
IMAGE EVALUATION TEST TARGET (QA-3)











APPLIED IMAGE . Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989