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**ISOLATION AND CHARACTERIZATION OF NISIN-PRODUCING
LACTOCOCCUS LACTIS SUBSP. *LACTIS* FROM BEAN SPROUTS**

A thesis submitted to the School of Graduate Studies

University of Ottawa

In partial fulfilment of the requirement for the degree of

Master of Science

Department of Microbiology & Immunology

Faculty of Medicine

By

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ABSTRACT

Bacterial isolates from bean-sprouts were screened for anti-*Listeria monocytogenes* bacteriocins using a well-diffusion method. Thirty-four isolates inhibited the growth of *L. monocytogenes*. Ten strains, which produced the biggest zones of inhibition, were characterized. All ten isolates were similar in that they had identical antibiograms, contained a common size 25 Kb plasmid (except for isolate 77) and produced similar bacteriocin. However, they could be placed into different groups by plasmid and pulsed-field gel electrophoresis (PFGE) typing. The ten isolates were presumptively identified as enterococci by the VITEK system. However, testing using an *Enterococcus* specific gene probe determined that these strains were not enterococci. One strain, isolate 80, which exhibited the strongest inhibition against *L. monocytogenes*, was selected for subsequent in-depth analysis. Both ribotyping and DNA sequencing of 16S ribosomal RNA demonstrated that the isolate was *Lactococcus lactis* subsp. *lactis*. In MRS broth, isolate 80 survived at 3 to 4.5°C for at least 20 days, grew at 4°C, and produced anti-listerial compounds at 5°C. When co-cultured with *L. monocytogenes* in MRS broth, the isolate inhibited the growth of *L. monocytogenes* at 4°C after 14 days and at 10°C after 2 days. Primary characterization showed that the anti-*L. monocytogenes* compound was proteinaceous (inactivated by proteinase K, protease, trypsin, α -chymotrypsin, pepsin and papain) and the antimicrobial effects were not caused by pH, a phage or H₂O₂, indicating that it was a bacteriocin. A plasmid cured derivative of isolate 32 was obtained by incubation at 40°C in MRS broth containing acridine orange. This plasmid-free strain still produced a bacteriocin, and had the same antibiotic resistance pattern as the wild type strain, indicating that the antibiotic

resistance and bacteriocin genes were most likely to be chromosomally mediated. This was confirmed by Southern hybridization of chromosomal DNA after the bacteriocin gene was identified. These bacteriocin-producing isolates had immunity to nisin, further suggesting that they produced nisin; a finding confirmed by PCR with primers specific for the nisin structural gene. Nucleotide sequencing of the PCR amplicon revealed that the form of nisin produced was nisin Z. When co-inoculated with 10^2 cells g^{-1} of *L. monocytogenes* on fresh-cut, ready-to-eat Caesar salads, bean-sprout isolate 80 (10^8 cells g^{-1}) was able to reduce the number of *L. monocytogenes* by 1 to 1.4 logs after storage for 10 days at 7 and 10°C. Inoculated together with a bacteriocin-producing *Enterococcus faecium* (10^8 cells g^{-1}), bean-sprout isolate 80 (10^8 cells g^{-1}) reduced the numbers of *L. monocytogenes* by 1.5 logs after storage for 10 days at 10°C and by 1 log after 10 days of storage at 7°C as compared to the control group. The results indicated that lactic acid starter cultures, possibly in combination with other hurdles, can be used to control the growth of *L. monocytogenes* in fresh-cut vegetables.

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TABLE OF CONTENTS

ABSTRACT.....	i
ACKNOWLEDGEMENTS.....	iii
DEDICATION.....	v
TABLE OF CONTENTS.....	vi
LIST OF TABLES.....	xi
LIST OF FIGURES.....	xii
LIST OF ABBREVIATIONS.....	xiii
1. INTRODUCTION.....	1
1.1 <i>Listeria monocytogenes</i>	1
1.1.1 Historical background.....	1
1.1.2 Taxonomy.....	1
1.1.3 Microbiology.....	2
1.2 Listeriosis.....	2
1.3 Control of listeriosis related to fresh-cut-ready-to-eat vegetables.....	3
1.3.1 Current control measures of listeriosis related to fresh-cut-ready-to-eat vegetables.....	3
1.3.2 Bacteriocin.....	5
1.3.3 Nisin and its use as a food preservative.....	5
1.3.4 Lactic acid bacteria and its as a food preservative.....	7
1.4 Hypothesis.....	9
1.5 Objectives and approaches.....	9

2.	MATERIALS AND METHODS.....	10
2.1	Bacterial strains and culture conditions.....	10
2.2	Isolation of bacteriocin-producing strains.....	10
2.3	Identification of bacteriocin-producing strains.....	12
2.4	Speciation of bacteriocin-producing strains.....	12
	2.4.1 Morphology.....	12
	2.4.2 Biochemical tests.....	12
	2.4.3 Hybridization with <i>Enterococcus</i> DNA probe.....	13
	2.4.4 Growth characteristics.....	13
	2.4.5 Ribotyping.....	14
	2.4.6 Sequencing of the 16S rRNA gene.....	14
2.5	Comparison of bean-sprout isolates.....	16
	2.5.1 Plasmid profile.....	16
	2.5.2 Antibiogram.....	17
	2.5.3 PFGE typing.....	18
2.6	Bacteriocin characterization.....	20
	2.6.1 Testing to exclude pH effects.....	20
	2.6.2 Testing to exclude phage effects.....	20
	2.6.3 Heat resistance.....	20
	2.6.4 Enzyme profiles.....	21
	2.6.5 Nisin immunity of bean-sprout isolates.....	21
	2.6.6 PCR amplification and sequencing of nisin gene.....	21

2.7	Location of the nisin Z gene.....	25
2.7.1	Curing of plasmids	25
2.7.2	Hybridization of nisin Z gene probe with genomic DNA.....	25
2.8	Copy number of nisin Z structural gene.....	26
2.9	Anti-listerial activity of bean-sprout isolates.....	26
2.9.1	Co-culture test in medium broth.....	26
2.9.2	Anti-listerial spectrum.....	27
2.9.3	Inoculation of bean sprout isolate onto fresh-cut-ready-to-eat vegetables.....	27
2.10	Statistical analysis.....	28
3.	RESULTS.....	30
3.1	Isolation and identification of bacteriocin-producing strains.....	30
3.2	Speciation of bacteriocin-producing strains.....	30
3.2.1	Morphology.....	30
3.2.2	Biochemical tests.....	30
3.2.3	DNA hybridization.....	31
3.2.4	Growth characteristics.....	31
3.2.5	Ribotyping.....	31
3.2.6	16S rRNA gene sequencing.....	36
3.3	Comparison of the bacteriocin-producing bean-sprout isolates.....	36
3.3.1	Plasmid profile.....	36
3.3.2	Antibiogram.....	36

3.3.3	PFGE typing.....	40
3.3.4	Summary of bean-sprout isolate comparison.....	40
3.4	Characterization of the anti-listerial compounds.....	44
3.4.1	pH effects.....	44
3.4.2	Phage effects.....	44
3.4.3	Heat resistance and enzyme profiles.....	44
3.4.4	Bacteriocin immunity.....	44
3.4.5	PCR amplification and sequencing of the nisin gene.....	49
3.5	Nisin gene location.....	49
3.5.1	Bacteriocin producing activity of the plasmid cured strain.....	49
3.5.2	Hybridization of the <i>nisZ</i> gene probe with genomic DNA of bean-sprout isolates.....	49
3.6	Nisin Z gene copy number.....	55
3.7	Anti-listerial activity of bean-sprout isolate 80.....	55
3.7.1	Anti-listerial spectrum.....	55
3.7.2	Comparison of anti-listerial activity of bean-sprout isolate 80 and bacteriocin-producing <i>E. faecium</i>	55
3.7.3	Inhibition of <i>L. monocytogenes</i> by bean sprout isolate <i>in vitro</i>	56
3.7.4	Inhibition of <i>L. monocytogenes</i> by bean sprout isolate in Caesar salad.....	56
3.7.5	Growth of lactic acid bacteria in Caesar salad.....	57
3.7.6	Headspace atmosphere of Caesar salad.....	57

3.7.7	Change of pH in Caesar salad.....	57
4.	DISCUSSION AND CONCLUSION.....	63
5.	LIST OF REFERENCES.....	76

LIST OF TABLES

1. The bacterial strains used in this study.....	11
2. Identification of bean-sprout isolates by the VITEK test.....	34
3. Identification of bean-sprout isolates using the <i>Enterococcus</i> specific DNA probe (AccuProbe™).....	35
4. Antibigrams of various bean-sprout isolates.....	39
5. Summary of phenotypic and molecular typing of bean-sprout isolates.....	43
6. The effect of pH neutralization on the anti-listerial activity of bean-sprout isolates supernatant	45
7. The effect of various enzymes and heat on the anti-listerial activity of bean-sprout isolate culture supernatants	47
8. Immunity of bean-sprout isolates against nisin producing <i>L. lactis</i> subsp. <i>lactis</i>	48
9. Anti-listerial spectrum of bean-sprout isolate 80.....	59
10. Comparison of the anti-listerial activity of the supernatant of bean-sprout isolate 80 and <i>E. faecium</i> ATCC 19434.....	60

LIST OF FIGURES

1. Location of the primers used for PCR analysis and sequencing of nisin gene.....	24
2. Inhibition of <i>L. monocytogenes</i> by <i>L. lactis</i> subsp. <i>lactis</i> isolated from bean-sprout as measured by the well-diffusion assay.....	32
3. Transmission electron micrograph of bean-sprout isolate 80.....	33
4. 16S rRNA gene alignment of bean-sprout isolate 80 and <i>L. lactis</i> subsp. <i>lactis</i>	37
5. Plasmid profiles of bean-sprout isolates.....	38
6. PFGE patterns from <i>SmaI</i> digested genomic DNA of bean-sprout isolates.....	41
7. Dendrogram of bean-sprout isolates generated from PFGE banding patterns.....	42
8. Flip-gel test.....	46
9. Amplification of the <i>nisZ</i> gene of bean-sprout isolates.....	50
10. Restriction analysis of PCR amplicons of the nisin gene from bean-sprout isolates.....	51
11. Alignment of DNA sequence of the nisin gene.....	52
12. Curing of plasmids from bean-sprout isolates.....	53
13. Southern hybridization of <i>nisZ</i> probe to <i>SmaI</i> digested genomic DNA of bean-sprout isolates.....	54
14. Southern hybridization of <i>nisZ</i> probe to <i>EcoRI</i> digested genomic DNA of bean-sprout isolates.....	58
15. Inhibition of <i>L. monocytogenes</i> by bean-sprout isolate 80 in buffered MRS broth.....	61
16. Inhibition of <i>L. monocytogenes</i> by bean-sprout isolate 80 in fresh-cut-ready-to-eat Caesar salad.....	62

LIST OF ABBREVIATIONS

A	adenine
bp	base pair(s)
C	cytosine
cfu	colony forming unit(s)
d	day(s)
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside 5'-triphosphate
EDTA	ethylenediaminetetra acetic acid
ESP	EDTA-sarcosine-proteinase K
FCV	fresh-cut-ready-to-eat vegetable(s)
FDA	Food and Drug Administration, USA
g	gram(s)
G	guanine
GRAS	generally recognized as safe
h	hour(s)
HPB	Health Protection Branch, Health Canada
Kb	kilobase(s)
kD	kilodalton(s)
LAB	lactic acid bacteria
LPM	lithium chloride, phenylethanol, moxalactam <i>Listeria</i> selective agar
M	molar(s)

mg	microgram(s)
min	minute(s)
mL	milliliter(s)
mm	millimeter(s)
MRS	De Man, Rogosa, Sharpe agar/broth
O/N	overnight
PCR	polymerase chain reaction
PALCAM	polymyxin-acriflavine-LiCl-ceftazidime-esculin-mannitol-<i>Listeria</i>-selective agar
PMSF	phenylmethylsulfonyl fluoride
PFGE	pulsed-field gel electrophoresis
PLU	photometric light unit
RNA	ribonucleic acid
RNAase	ribonuclease
rRNA	ribosomal ribonucleic acid
TSB-YE	tryptone soya broth containing 0.6% yeast extract
U	unit
UHT	ultra-high temperature
UPGMA	unweighted pair group method using arithmetic averages
vol	volume
wt	weight
µg	microgram(s)

μL

microliter(s)

1. INTRODUCTION

1.1 *Listeria monocytogenes*

1.1.1 Historical background

L. monocytogenes was first described and named by Murray *et al.* (1926) as *Bacterium monocytogenes*. In 1927, the bacterium was isolated from the livers of several gerbils and was named *Listerella hepatolytica* in honour of Dr. J. Lister, and renamed as *L. monocytogenes* in 1940 (Gray and Killinger, 1966). The first confirmed report of listeric infection in humans appeared in 1929, and soon after that, *L. monocytogenes* was established as a causal agent of meningitis and perinatal infection (Bahk and Marth, 1990). In 1985, *L. monocytogenes* caused a large (142 cases with 48 deaths) foodborne outbreak in California (Linnan *et al.*, 1988). Since then, the organism has been a source of continual problems for the food industry and regulatory agencies.

1.1.2 Taxonomy

L. monocytogenes belongs to the genus *Listeria*, which is listed in Group 19 in the ninth edition of the Bergey's Manual together with *Brochothrix*, *Lactobacillus*, *Kurthia*, *Renibacterium*, *Caryophanon*, *Erysipelothrix* and *Carnobacterium*, is referred to as "regular, nonsporing, Gram-positive rods" (Holt *et al.*, 1994a). Based on numerical taxonomic studies, Farber and Peterkin (1991) concluded that the genus *Listeria* includes the species *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. ivanovii*, *L. grayi*, and *L. murrayi*. This genus is closely related to the genus *Brochothrix*; both of these genera occupy a position between *Lactobacillus* and *Bacillus* and are more distantly related to *Streptococcus*, *Lactococcus*, *Enterococcus*, *Staphylococcus*, *Kurthia*, *Gemella*, and

Erysipelothrix.

1.1.3 Microbiology

L. monocytogenes is a Gram-positive, nonsporeforming, facultatively anaerobic rod. *Listeria* spp. are psychrotrophic organisms, capable of growing at refrigeration temperatures. Therefore, they are a concern in refrigerated foods. The actual temperature range for growth is between -0.4 and 50°C, with the optimum being 25-30°C (Juntilla *et al.*, 1988; Walker and Stringer, 1987). Walker *et al.* (1990) examined the growth of three strains of *L. monocytogenes* in chicken broth and/or ultra-high temperature (UHT) milk. In both media, lag and generation times were 3 to >34 d and 62-131 h at 0°C, and 1-3 d and 13-24 h at 5°C, respectively. The organism grows over a wide temperature range in very acidic environments (pH value as low as 4.3), as well as in the absence of, or very low amounts of O₂ (Farber *et al.*, 1996; Farber and Peterkin, 1991; Wimpfheimer *et al.*, 1990). These properties enable the organism to multiply in a wide range of environments, including foods.

The organism is very widespread in the environment and can be found in many different locations such as water, silage, sewage, slaughterhouse waste, milk of normal and mastitic cows, as well as human and animal feces (Farber and Peterkin, 1991; Van Renterghem *et al.*, 1991; MacGowan *et al.*, 1994).

1.2 **Listeriosis**

Although the sources and route of infection of the disease need further investigation, contaminated food is the primary source of the organism (Farber and Peterkin, 1991). The virulence (Portnoy *et al.*, 1992; Rocourt, 1994; Menudier *et al.*, 1991) and health risks associated with different species and strains have been discussed (Hof and Rocourt, 1992),

concluding that *L. monocytogenes* is the species of most concern, although the haemolytic species *L. seeligeri* and *L. ivanovii* are also recognized as potential pathogens (Menudier *et al.*, 1991; Lessing *et al.*, 1994). The infectious dose for humans is unknown and may vary widely depending on several factors, of which the immune status of the individual and the virulence of the strain ingested are probably the most important (Farber and Peterkin, 1991).

Most cases of human listeriosis appear to be sporadic, although a portion of these sporadic cases may be previously unrecognized common-source clusters (Ciesielski *et al.*, 1988). High risk individuals for acquiring listeriosis include the immunosuppressed, elderly, pregnant and those suffering a range of underlying diseases. However, otherwise healthy individuals may also acquire listeriosis (Farber and Peterkin, 1991). The spectrum of the disease in humans is broad, ranging from asymptomatic infection and carriage to uncommon cutaneous lesions, flu-like symptoms, various focal infections such as septic arthritis, conjunctivitis, urethritis, endocarditis, and the more serious and better recognized conditions of septicemia, abortion and meningoencephalitis (Farber and Peterkin, 1991).

1.3 Control of listeriosis related to fresh-cut-ready-to-eat vegetables (FCV)

1.3.1 Current control measures of listeriosis related to FCV

L. monocytogenes is known to be present on a wide range of plant materials (Beuchat *et al.*, 1990), and is often found in food-processing environments, on the hands of food workers (Genigeogis *et al.*, 1990; Kerr *et al.*, 1993), and as potentially dangerous biofilms on food-processing equipment (Mafu *et al.*, 1990; Zottola and Sasahara, 1994). As a result of recurring outbreaks and the formidable characteristics possessed by *L. monocytogenes* (psychrotroph, high mortality rate, widespread, heat resistance), U.S. federal regulatory

agencies have adopted a "zero tolerance" policy for *L. monocytogenes* in ready-to-eat foods (Crawford, 1989; Klima and Montville, 1995).

Minimally-processed or fresh-cut vegetables are one of the ready-to-eat foods, which have become an economically important convenience item (Wiley, 1994). The processing of FCV includes washing, sanitizing, cutting and packaging (Wiley, 1994). The rich substrate resulting from the cutting of the vegetables can support the growth of foodborne pathogens, including *L. monocytogenes* (Aytac and Gorris, 1994; Berrang *et al.*, 1989). There have been reports of some foodborne outbreaks where FCV have been implicated (Nguyen-the and Carlin, 1994), and this has led to a search for novel methods of controlling foodborne pathogens. Chlorination, which has been found to have limited effectiveness, is the major sanitizer currently being used in the FCV industry (Zhang and Farber, 1996). Therefore, alternate or extra hurdles to the growth or survival of *L. monocytogenes* in FCV are urgently needed.

Naturally-occurring antimicrobial substances, e.g., compounds in carrot juice have been found to inactivate *Listeria*, but their usefulness is limited by temperature (Beuchat and Doyle, 1995). The efficacy of a wide variety of chemical disinfectants has recently been tested, with all only causing approximately a 1-log reduction in numbers of *L. monocytogenes* on FCV (Zhang and Farber, 1996). Another approach to control *L. monocytogenes* is the use of either lactic acid bacteria (LAB) or bacteriocins. The latter approach has attracted increased attention since nisin was accepted by the U.S. Food and Drug Administration (FDA) in 1987 as a generally recognized as safe (GRAS) food additive for dairy products.

1.3.2 Bacteriocin

Bacteriocins, as defined by Tagg *et al.* (1976), are proteinaceous compounds that inactivate bacteria which are closely related to the producer organism. While true for the majority of compounds, it is now evident that bacteriocins may take many forms and elicit bactericidal activity beyond species that are closely related, or confined within the same ecological niche. Bacteriocins are directly produced as ribosomally synthesized polypeptides or precursor polypeptides. This is different from the antibiotic peptides, e.g., bacitracin, gramicidin and valinomycin, which are synthesized by multienzyme complexes. The biosynthesis of these latter compounds, in contrast to that of bacteriocins, is not directly blocked by inhibitors of ribosomal protein synthesis (Jack *et al.*, 1995). As described by Klaenhammer (1993), there are four distinct classes of bacteriocins: i) lantibiotics, small membrane-active peptides (<5 kD) containing the unusual amino acid lanthionine; ii) small heat-stable, non-lanthionine containing membrane-active peptides (<10 kD); iii) large heat-labile proteins (>30 kD); and iv) complex bacteriocins, composed of protein plus one or more chemical moieties (lipid, carbohydrate) required for activity.

1.3.3 Nisin and its use as food preservative

Nisin is a lantibiotic containing 34 amino acids, including some unusual amino acid residues such as dehydroalanine, dehydrobutyrine, lanthionine and β methy-lanthionine (Jack *et al.*, 1995). It is produced by certain strains of *Lactococcus lactis* subsp. *lactis*, gram-positive cocci commonly found in milk. There are two forms of nisin, nisin A and nisin Z. Nisin Z is a naturally occurring variant of nisin, differing only in the exchange of asparagine for histidine at position 27 as the result of a single-base substitution (C to A) in the nisin

structural gene. Nisin Z is significantly more water soluble and heat stable at elevated pH values, and shows better diffusion properties in solids than nisin A, yet retains comparable biological activity (Jack *et al.*, 1995).

The nisin gene is in a cluster located in a 70 kb conjugative transposon *Tn5276* on the chromosome (Rauch and de Vos, 1992; Mulders *et al.*, 1991; Rodriguez *et al.*, 1995). Based on the current knowledge on the genetics of nisin, a model for nisin biosynthesis was developed by Entian and de Vos (1996) describing the function of the nisin gene cluster. The nisin gene cluster includes *nisA* or *nisZ* and *nisBCEFGIKPRT* with the gene *nisA* or *nisZ* being the structural gene. The genes *nisEFGI* encode nisin immunity-related proteins. The proteins NisBCPT are related to nisin modification and transportation, while NisKR may have the function of acting as a signal transducer for activating the nisin gene.

Nisin was first discovered in the late 1920s and early 1930s (Delves-Broughton *et al.* 1996). It was discovered as a result of investigations begun in the 1930s into the inhibitory effects on conventional starter cultures by a toxin present in milk. Inhibitory strains of *Lactococcus lactis* subsp. *lactis* were identified by Mattick and Hirsch in 1944 and the name 'nisin' was assigned to a substance characterized by the same workers in 1947 (Mattick and Hirsch, 1947). Nisin has long been known to have anti-microbial activity against a range of gram-positive vegetative cells and spores (Hurst, 1981). More recently, it has been shown that gram-negative and resistant gram-positive bacteria can become susceptible to nisin after sub-lethal treatments such as freezing, heating or exposure to weak acids or chelating agents (Delves-Broughton *et al.* 1996; Stevens *et al.*, 1991; Kalchayanand *et al.*, 1992).

Nisin was investigated as a potential food preservative by Hirsch *et al.* in 1951, and as a preservative in processed cheese in 1952 (McClintock *et al.* 1952). The commercial development of the nisin preparation called Nisaplin was carried out by Aplin & Barrett in 1957. Nisin was shown to be non-toxic by various workers in 1962 (Frazer *et al.* 1962; Hara *et al.* 1962). Commercial nisin concentrates were defined in terms of identity and purity by the Joint FAO/WHO Expert Committee on Food Additive (JECFA) in 1969 (WHO 1969) and the creation of an International Reference Preparation of Nisin by WHO in 1970. Nisin is currently approved as a food preservative in over 50 countries including the EEC (nisin's designated food additive number is E234) and the USA (Delves-Broughton, 1990).

Nisin has been used in a range of food products, particularly dairy products, canned vegetables and alcoholic beverages (Delves-Broughton, 1990), but there are no reports of its use with FCV.

1.3.4 Lactic acid bacteria as a food preservative

Most of the bacteriocins identified so far are expressed by lactic acid bacteria (LAB). Lactic acid bacteria comprise a diverse group of gram-positive, non-spore-forming bacteria belonging to the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Carnobacterium*, *Streptococcus*, *Lactococcus*, *Enterococcus*, *Vagococcus*, *Aerococcus*, *Alloiococcus*, *Tetragenococcus*, *Atopobium* and *Bifidobacterium* (Schleifer and Ludwig, 1995). They occur as cocci or rods and generally lack catalase, although pseudo-catalase can be found in rare cases. They are chemo-organotrophic and grow only in complex media. Fermentable carbohydrates are used as an energy source. Hexoses are degraded mainly to lactate (homofermentative) or to lactate and additional products such as acetate, ethanol, CO₂,

formate or succinate (heterofermentatives) (Schleifer and Ludwig, 1995). Lactic acid bacteria have been found in foods (dairy products, fermented meat, sour dough, fermented vegetables, silage, beverages), on plants, in sewage, but also in the genital, intestinal and respiratory tracts of man and animals (Hammes et al., 1991). The LAB and/or their bacteriocins have been extensively examined for controlling foodborne pathogens because of their widespread use as food starter cultures. Many applications for the use of bacteriocins in the control of foodborne pathogens on foods, e.g., meat, fish and dairy products have been developed (Muriana, 1996; Olasupo *et al.*, 1994; Degnan *et al.*, 1994). Recently, a number of drawbacks in using purified bacteriocins in food preservation have been noticed, i.e., poor solubility, sensitivity to food enzymes and uneven distribution in foods, often requiring the addition of high levels of bacteriocins. Instead of using purified bacteriocin, inoculating bacteriocin producing starter cultures may be more effective against foodborne pathogens. Mathieu *et al.* (1994) indicated that the starter culture *Carnobacterium piscicola* CP5 had much stronger inhibitory effects than partially purified carnocin CP5 against *L. monocytogenes* in skim milk. Vescovo *et al.* (1995) reported that the addition of lactobacilli and pediococci had a remarkable inhibitory effect on the growth dynamics of the microflora associated with ready-to-eat vegetables during refrigerated storage, indicating that the inoculation of ready-to-eat vegetables with selected lactic acid bacteria may be effective in controlling the growth of undesirable bacteria.

Although there have been no reports of the isolation of bacteriocin-producing LAB from fresh vegetables, Aytac (1994) found that mung bean sprouts which inhibited the growth of *L. monocytogenes* contained high levels of LAB.

1.4 Hypothesis

Based on the report that mung-bean sprouts have anti-listerial activity and contain high levels (10^6 - 10^9 cfu g⁻¹) of LAB, this study hypothesized that mung-bean sprouts contained bacteriocin producing lactic acid bacteria which had anti-listerial activity. This study further hypothesized that the bacteriocin-producing LAB from bean-sprouts could be used to inhibit the growth of *L. monocytogenes* in fresh-cut-ready-to-eat vegetables.

1.5 Objectives and Approaches

The overall objective of this project was 1) to isolate and characterize bacteriocin-producing lactic acid bacteria from bean-sprouts and 2) use these isolates to improve the safety of fresh-cut-ready-to-eat vegetables by inhibiting, reducing or eliminating the foodborne pathogen *L. monocytogenes*.

The specific objectives and approaches used in this study included:

- 1) Isolating bacteriocin-producing lactic acid bacteria from bean sprouts.
- 2) Characterizing the bacteriocin-producing isolates.
- 3) Characterizing the bacteriocin expressed by the isolated lactic acid bacteria.
- 4) Evaluating the effectiveness of the isolates in inhibiting the growth of *L. monocytogenes in vitro* and on fresh-cut-ready-to-eat vegetables.

2. MATERIALS AND METHODS

2.1 Bacterial strains and culture conditions

A description of the lactococci, enterococci, *Listeria* isolates and reference plasmids used in this study is given in Table 1. All lactic acid bacteria were lyophilized, as well as stored at -70°C in MRS broth with 40% glycerol. Other bacteria were stored at -70°C in tryptone soya broth (Oxoid, Nepean, Ont) containing 0.6% yeast extract (Difco Laboratories, Detroit, Michigan) (TSB-YE) with 40% glycerol. Frozen stock cultures of lactic acid bacteria were inoculated into MRS broth before each experiment. Frozen stock cultures of *L. monocytogenes* were streaked onto tryptose agar (Difco), and an isolated colony was transferred to TSB-YE before each experiment. All cultures were incubated at 30°C, except the enterococci which were incubated at 35°C.

2.2 Isolation of bacteriocin-producing strains.

Lactic acid bacteria were isolated from one batch of each unpackaged mung- and soy-bean sprouts which had been purchased from a retail store. Twenty-five grams of samples were placed into a plastic Stomacher™ bag containing 250 mL of 0.1% buffered peptone water (Oxoid) and were pummelled with a Stomacher™ (Lab-Blender 400, Seward Medical UAC House, London, England) for 3 min. Different dilutions of the samples were then plated onto De Man, Rogosa, Sharpe (MRS) agar (Oxoid) designed to encourage the growth of the lactic acid bacteria. Colonies with different morphological types were selected and purified on MRS agar.

Table 1. The bacterial strains used in this study

Species	Strain no.	Description/Source	Reference
<i>Lactococcus lactis</i> subsp. <i>lactis</i>	32	bean-sprout isolate, nisin+	This study
"	40	"	"
"	42	"	"
"	47	"	"
"	52	"	"
"	54	"	"
"	72	"	"
"	74	"	"
"	77	"	"
"	80	"	"
"	32j	plasmid-less derivative of bean-sprout isolate 32, nisin+	"
"	NCK400	sauerkraut, nisin+	Harris <i>et al.</i> (1992)
"	NCK402	derivative of NCK400, nisin-	"
<i>Escherichia coli</i>	K12 C600RA	contained plasmid RA1 (130.3 Kb)	Mendez <i>et al.</i> (1980)
"	K12 C600R	contained plasmid R222 (90.9 Kb)	"
"	K12 C600RP	contained plasmid RP1 (57.6 Kb)	"
<i>Enterococcus gallinarum</i>	ATCC35038	type strain for species reference	Collins <i>et al.</i> (1984)
<i>Enterococcus faecium</i>	ATCC19434	"	Schleifer and Kilpper-Bälz (1984)
<i>Listeria monocytogenes</i>	HPB3 (Scott A)	clinical isolate, serotype 4b	Zhang and Farber (1996)
"	HPB2 (81-861)	coleslaw isolate, serotype 4b	"
"	HPB534	beet isolate, serotype 1	"
"	HPB540	green bean isolate, serotype 1	"
"	HPB845	cabbage isolate, serotype 4	"

2.3 Identification of bacteriocin-producing strain

Bacteriocin production was determined using a well-diffusion methods described by Hoover and Harlander (1993). Tryptone soya agar (15 mL; Oxoid) containing 0.6 % yeast extract (Difco) and 1% disodium β -glycerophosphate (Sigma Chemical CO., St. Louis, MO) was liquefied (47.5°C) and inoculated with an overnight culture of *L. monocytogenes* strain Scott A (1%), a commonly used strain by researchers in the field, as an indicator organism. Then the agar was poured into a petri dish. After the agar was solidified, an 8-mm well was cut out, to which 100 μ L filtered supernatant of the strains to be tested was added. After incubation at 30°C for 24 h, the plates were examined for clear zones of inhibition surrounding the wells.

2.4 Speciation of bacteriocin producing strains

2.4.1 Morphology

The isolates were examined for morphological characteristics under the compound microscope after doing the Gram stain. In addition, the isolates were also examined in a Zeiss EM902 transmission electron microscope (Carl Zeiss, Thornwood, N.Y.) for the presence of flagella, using ammonium molybdate negative staining (Austin *et al.*, 1990).

2.4.2 Biochemical test

Biochemical testing was done using the Automated Bacterial Identification System VITEK-AMS model 60 with a VITEK Gram-positive identification card (Product No. V 1305; bioMérieux VITEK, Inc., Hazelwood, Missouri). The system identified the bacteria by comparing the database information with the results of tests, including arabinose, arginine, bacitracin, catalase, cellobiose, dextrose, esculin, hemicellulase, inulin, lactose,

mannitol, melezitose, melibiose, novobiocin, optochin, peptone base, pullulan, pyruvate, raffinose, ribose, salicin, sorbitol, sucrose, β -haemolysis, tetrazolium red, trehalose, urea, 10% bile, 40% bile, xylose and 6% NaCl. The Vitek database for the GPI card includes species of *Streptococcus*, *Staphylococcus*, *Enterococcus*, *Aerococcus*, *Listeria*, *Corynebacterium* and some other clinically significant bacteria, including *Erysipelothrix*, *Rhusiopathiae*, *Actinomyces pyogenes* and *Arcanobacterium haemolyticum*.

2.4.3 Hybridization with *Enterococcus* DNA probe

DNA hybridization was performed to confirm the identification by the VITEK test. Since the bean-sprout isolates were identified as enterococci by using the VITEK system, the organisms were tested with an *Enterococcus* Culture Identification Test Kit (*Enterococcus* Accuprobe™, Gene Probe, San Diego, CA) using the supplier's protocol and reagents. This test kit is based on the hybridization of DNA from lysed bacterial cells with DNA probe specific for enterococcal rRNA sequences.

2.4.4 Growth characteristics tests

DNA hybridization results differed from those obtained with the VITEK system. It was later found that the database does not provide enough biochemical information to properly identify lactic acid bacteria. Therefore, further tests, as follows, were performed to identify the isolates.

a) Isolates were tested for their ability to grow in the presence of 4% NaCl using MRS broth as a base.

b) MRS broth was inoculated and incubated at 3 to 13°C using a temperature gradient incubator (Scientific Industries Inc., Mineola, N.Y.), as well as 45°C, to determine

the ability to grow at low and elevated temperatures. At the end of the low temperature growth experiments, the supernatants of cultures grown at different temperatures were tested for anti-listerial activity using a well-diffusion method.

2.4.5 Ribotyping

Ribotyping was performed using the RiboPrint[®] system (DuPont Central Research and Development Experimental Station, Wilmington, DE). The method was reported to be able to provide accurate species and type description of lactic acid bacteria, as well as foodborne pathogens (Webster, 1995). Isolates 80 and 32 were sent to the DuPont Central Research and Development Experimental Station, by using the RiboPrint[®] system. Bacterial cells were lysed, DNA fragments generated using *EcoRI* restriction endonuclease and patterns created using a labelled ribosomal RNA operon. The patterns were imaged using chemiluminescence and photographed. Through the use of algorithms for data normalization, patterns were obtained. Visual comparisons among pattern sets for all strains evaluated were made to determine relatedness among strains; strains with similar banding patterns were grouped into clusters.

2.4.6 Sequencing of the 16S rRNA gene

a) Nucleic acid preparation. Chromosomal DNA was isolated as described by Leenhouts *et al.* (1989).

b) PCR amplification and purification of PCR product. PCR amplification was performed using the procedures described by Weisburg *et al.* (1991). Genomic DNA (1.5 µg) was amplified in a 50-µl reaction in a thermocycler (Gene Amp PCR System 9600, Perkin Elmer, Norwalk, CT). The primers used were:

Fd1. 5'-AGAGTTTGATCCTGGCTCAG

Rd1. 5'-AAGGAGGTGAATCCAGCC

Conditions consisted of 35 cycles of 95°C (2 min), 42°C (30 sec), and 72°C (4 min), plus one additional cycle consisting of a final 20 min chain elongation. The amplified products were electrophoresed in a 1.5% gel of high strength analytical grade agarose (Bio-Rad Laboratories, Hercules, CA). The amplicon band was excised from the gel and purified using Wizard™ PCR Preps DNA purification system (Promega Corporation, Madison, WI), according to manufacturer's instructions.

c) Sequencing methods. The purified PCR products were further purified prior to sequencing with a QIA quick-spin PCR Purification Kit (Qiagen Inc, Chatsworth, CA), and then sequenced using a PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems Inc, Toronto, Ont) in a Perkin Elmer Cetus Model 480 Cycler (Perkin Elmer), following the protocol supplied by the manufacturer. The cycle sequencing products were purified using Centri-Sep™ spin columns (Princeton Separations, Inc, Adelphia, NJ), and the purified samples were loaded onto an Applied Biosystems 373A DNA sequencer (Applied Biosystems). The sequencing data was collected and analyzed with the Sequencing Analysis Software associated with the sequencer.

d) Sequencing Primers. Both forward and reverse sequencing primers useful for conserved regions within 16S rRNA genes, have been described previously (Lane *et al.*, 1985; Weisburg *et al.*, 1989). The forward primers used in this study spanned the following position (*Escherichia coli*) numbers:

Fd1. 8-27, 5'-AGAGTTTGATCCTGGCTCAG

Fd2.	339-357,	5'-CTCCTACGGGAGGCAGCAG
Fd3.	785-805,	5'-GGATTAGATTACCCTGGTAGTC
Fd4.	907-926,	5'-AAACTCAAATGAATTGACGG
Fd5.	1391-1406,	5'-TGTACACACCGCCCGT

Reversed primers include:

Rd1.	1541-1525,	5'-AAGGAGGTGAATCCAGCC
Rd2.	1513-1494,	5'-TACGGTTACCTTGTTACGAC
Rd3.	1406-1392,	5'-ACGGGCGGTGTGTACA
Rd5.	926-907,	5'-CCGTCAATTCATTTGAGTTT
Rd6.	802-785,	5'-TAGGTCCCATAGATTAGG
Rd7.	536-519,	5'-GTATTACCGCGGCTGCT
Rd8.	357-342,	5'-CTGCTGCCTCCCGTAG

e) Analysis of the 16S rRNA gene sequence results. The sequence results were sent to GenBank for an homology sequencing search using Blast (Altschul *et al.*, 1990). The data base searched was Blastn 1.4.8 MP (Built at 08:41:09 Oct 19, 1995). Based on the searching results, similar 16S rRNA gene sequences were aligned with that of the bean-sprout isolate 80 using the PC/Gene[®] computer program (IntelliGenetics, Inc., Mountain View, CA).

2.5 Comparison of bean-sprout isolates

2.5.1 Plasmid profile

Plasmid DNA was extracted by a modified procedure of Birnboim and Doly (1979), and a modified Wizard[™] Minipreps DNA purification system (Promega, Madison, WI).

a) Modified Birnboim and Doly 's method: Plasmid DNA were isolated using the

protocol developed by Birnboim and Doly (1979), except a treatment of proteinase K (0.5 mg mL⁻¹; Boehringer Mannheim, Mississauga, Ont) at 50°C for 30 min was included before alkaline SDS lysis of the cells.

b) Modified method for the Wizard™ Minipreps DNA Purification Systems. The cell resuspension solution was replaced with a solution containing lysozyme, which consisted of sucrose (25%; Sigma), lysozyme (5 mg mL⁻¹; Sigma) and ribonuclease (0.1 µg mL⁻¹; Boehringer Mannheim). The resuspended cells were incubated for 12 min at 37°C. A treatment of proteinase K (0.5 mg mL⁻¹; Boehringer Mannheim) at 50°C for 30 min was included before adding the cell lysing solution.

Plasmids were separated by electrophoresis in a 0.8% gel of high strength analytical grade agarose (Bio-Rad) at 4.5 V cm⁻¹ for 2 h, and detected with ethidium bromide (0.5 µg mL⁻¹, Sigma; Sambrook *et al.*, 1989a). The results were recorded with an image system, Gel Print 2000i BioPhotonics (Bio/Can Scientific, Mississauga, Ont) under UV transillumination and interpreted using the Molecular Analyst/PC Fingerprinting Software version 1 (Bio-Rad). The plasmid sizes were determined by isolating and analysing the plasmids at least three times. The plasmid sizes in fig. 5 are average sizes.

2.5.2 Antibiogram

Antibiotic resistance profiles of the isolates were determined by using the Dispens-O-Disc™ Susceptibility Test System (Difco), a disk diffusion test system. The tests were performed using the supplier's protocol, interpretive standards and quality control limits. Isolates were inoculated into 5 mL MRS broth and incubated at 30°C for 4 h. Then 0.1 mL of the broth was added to 5 mL tryptone soya broth (Oxoid), and incubated with shaking at

30°C until an OD₆₂₅ of 0.08-0.1 was reached (about 4 h). The cultures were swabbed onto Muller-Hinton agar (Oxoid), and antibiotic disks (Difco) were then placed onto the plates. After incubation at 30°C for 24h, the diameter of the zone of inhibition around the disk was measured. *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923) and *Pseudomonas aeruginosa* (ATCC 27853) were used as control strains.

The common antibiotics against Gram-positive bacteria were chosen for the antibiogram analysis. Antibiotics included: ampicillin (10 µg), cefotaxime (30 µg), chloramphenicol (30 µg), gentamicin (10 µg), polymyxin B (300 U), streptomycin (10 µg), trimethoprim (1.25 µg)-sulfamethoxazole (23.75 µg), tetracycline (30 µg), sulfisoxazole (300 µg), cephalothin (30 µg), and nalidixic acid (30 µg). Because the isolates were initially identified as enterococci, vancomycin (30 µg) resistance was also tested.

2.5.3 Pulsed-field gel electrophoresis (PFGE) typing

PFGE of *Sma*I digested genomic DNA of the isolates was performed using the method described by Tanskanen *et al.* (1990).

a) Preparation of genomic DNA *in situ* in agarose blocks. An overnight MRS broth culture of the various isolates was diluted (1:100) in fresh MRS broth and grown at 30°C to an OD (A₆₀₀) of 0.6 (approximately 2.5 h, shaking at 200 rpm.). The cells (from 1.5 mL of culture) were harvested by centrifugation at 16,000 x g for 1 min in a MicroMax Eppendorf microcentrifuge (IEC, Needham HTS, MA), washed with 1.5 mL of 1 M NaCl (Sigma)-10 mM Tris HCl (pH 7.6; Sigma), and resuspended in 300 µl of 1 M NaCl-10 mM Tris HCl (pH 7.6). The suspension was then mixed with 300 µl of 2% (wt/vol) low melting point agarose (GIBCO BRL), and dispensed into 6 plastic insert moulds (Pharmacia Biotech Inc., Baie

d'Urfé, Québec). The cells were lysed *in situ* for 18 h at 37°C with 10 mL EC buffer (6 mM Tris HCl [pH 7.6], 1 M NaCl, 100 mM disodium EDTA [pH 7.6; Sigma], and 1% [wt/vol] sarcosine [Sigma]) containing 10 mg of lysozyme (1 mg mL⁻¹). The buffer was replaced with 8 mL fresh EDTA-sarcosine-proteinase K (ESP) solution (0.25 M EDTA, pH 8.0; 0.5% sarcosine; 0.5 mg mL⁻¹ proteinase K) and incubated at 50°C for 48 h. The agarose blocks were treated four times within 2 h (25°C) with 1 mM phenylmethylsulfonyl fluoride (PMSF) in TE buffer (10 mM Tris HCl [pH 8.0], 1 mM disodium EDTA), and then washed four times with TE buffer over a 2 h period. The agarose blocks were digested directly or stored at 4°C in 0.5 M sodium EDTA (pH 8.0) and 1% (wt/vol) sarcosine until required for digestion.

b) Digestion of DNA in agarose blocks. The agarose blocks were washed three times for 1 h with TE buffer, and then sliced to a thickness of 1 to 2 mm. The slices were incubated for 18 h at 25°C with 50 U of *Sma*I (Boehringer Mannheim) in 100 µl buffer recommended by the supplier.

The *L. lactis* chromosome has a low G+C content of 36.8 to 37.3% (Kilpper-Bälz *et al.* 1982). The recognition sequences of *Sma*I are rich in G and C nucleotides (CCC↓GGG), and therefore should digest the *L. lactis* chromosome infrequently, yielding a small number of large fragments that may be separated by PFGE.

c) PFGE. The DNA agarose slices were loaded into a 1% gel of high strength analytical grade agarose (Bio-Rad), and electrophoresed at 15°C in 0.5 x TBE buffer solution (Boehringer Mannheim; 45 mM Tris, 45 mM boric acid, pH 8.3, and 1 mM sodium EDTA) for 18 h at 200 V in a Bio-Rad CHEF DR® II electrophoresis cell. The pulse time was

increased linearly from 1 to 20 sec. The DNA was visualized by staining with ethidium bromide ($0.5\mu\text{g mL}^{-1}$, Sambrook *et al.*, 1989a). The results were recorded using the Gel Print 2000i BioPhotonics image system under UV transillumination and interpreted using the Molecular Analyst/PC Fingerprinting Software version 1.

2.6 Bacteriocin characterization

2.6.1 Testing to exclude pH effects

Overnight culture supernatant was neutralized with 1 N NaOH to pH 7, then tested for anti-listerial activity by using the well-diffusion method (Hoover and Harlander, 1993).

2.6.2 Testing to exclude phage effects

Since phages are not motile, and bacteriocins can diffuse, a flip-streak method (Hoover and Harlander., 1993) was used to exclude the possible effects of an anti-listerial phage. A loopful of glycerol frozen culture of bean-sprout isolate 80 was streaked down the middle of an MRS agar plate and then incubated overnight at 30°C . Then, a sterile spatula was used to dislodge the agar from the bottom of the petri plate, and then to flip it over onto the cover. A loopful of overnight TSB-YE broth culture of *L. monocytogenes* Scott A was then streaked onto the inverted agar perpendicular to the streak of the isolates. After incubation at 30°C for 24 h, the plates were checked for a clear zone parallel to the streaked isolate 80.

2.6.3 Heat resistance

The supernatant from an overnight culture was heated at 60°C for 30 min, 100°C for 10 min or autoclaved (125°C , 15 min). The well-diffusion method was used to determine

the anti-listerial activity of the treated supernatants.

2.6.4 Enzyme profiles

The supernatant (400 μ l) from an overnight culture was incubated for 2 h at 37°C with 80 μ l of various enzyme solutions (5 mg mL⁻¹), and then boiled for 5 min. The well-diffusion method was used to determine the anti-listerial activity of the treated supernatant. The enzymes used were as follows: pronase E (Sigma), trypsin (Sigma), α -chymotrypsin (Sigma), papain (Sigma), pepsin (Sigma), catalase (Sigma) and proteinase K (Boehringer Mannheim). All enzymes except pepsin were diluted in 10 mM sodium phosphate buffer (Sigma), pH 6.8. Pepsin was diluted in 0.02 N HCl, pH 2.0.

2.6.5 Nisin immunity of bean sprout isolates

Based on the enzyme profiles, the heat resistance characteristics and gene location of the anti-listerial compound(s), nisin immunity tests were performed with a stabbing method (G. LaPoint, personal communication). Tryptone soya agar (15 mL; Oxoid) containing 0.6 % yeast extract (Difco) and 1% disodium β -glycerophosphate (Sigma Chemical CO., St. Louis, MO) were liquefied and kept at 47.5°C. The agar was inoculated with a overnight culture of indicator strains (1%) and poured into a petri dish. The bean-sprout isolates and *L. monocytogenes* strain Scott A were used as indicator strains . After the agar was solidified, a colony of nisin producing *L. lactis* subsp. *lactis* NCK400 was stabbed in to the agar. Inhibition zones were measured after 24 h of incubation at 30°C.

2.6.6 PCR amplification and sequencing of nisin gene

The bean-sprout isolates were found to have nisin immunity, indicating that the anti-listerial compounds were possibly nisin. To further confirm this finding, experiments could

have been done to purify and sequence the bacteriocin, or characterize its coding gene. Since nisin genes have been well characterized (Engelke *et al.*, 1994; Kuipers *et al.*, 1993; Mulders *et al.*, 1991; Rauch and de Vos, 1992), and specific PCR primers have been designed (Rodrigues *et al.* 1995), PCR amplification and sequencing of the bacteriocin gene were performed to identify the bacteriocin expressed by the bean-sprout isolates.

a) Polymerase chain reaction of the nisin structural gene. Overnight MRS broth cultures of the isolates were pelleted and suspended with double distilled water to a final concentration of 10^6 cells μl^{-1} . The cells (10 μl) were lysed at 94°C for 8 min and then used directly as template for a 50- μl PCR reaction in a thermal cycler (Gene Amp PCR System 9600, Perkin Elmer). Conditions used for the PCR were modified from those described by Horn *et al.* (1991), with the annealing temperature being 42 instead of 35°C. Increasing the annealing temperature during the PCR significantly reduced nonspecific amplification. The modified conditions consisted of 35 cycles of 94°C for 1 min, 42°C for 1 min and 72°C for 1.5 min, plus one additional cycle of 72°C for 5 min. The primers used in this study were designed by Rodriguez *et al.* (1995) and contained the following sequences:

Primer 1, 5'-TTGGTATCTGTTTCGAA

Primer 2, 5'-CCATGTCTGAACTAACA

Primer 3, 5'-CGCGAGCATAATAAACGGCT

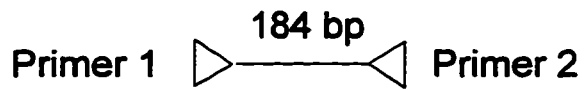
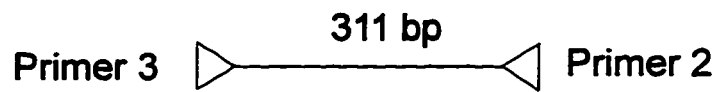
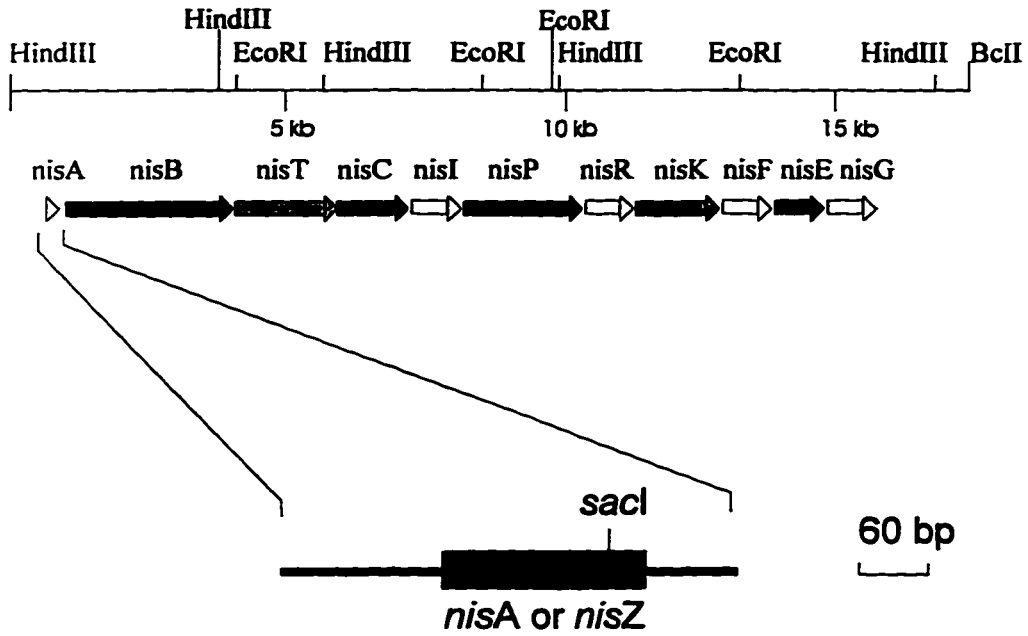
PCR using the above primers amplified part of or the whole sequence of nisin structural gene (*nisA* or *nisZ*) which is located in a 70-Kb chromosomally-located conjugative transposon, Tn5276 (Rauch and de Vos, 1992; Mulders *et al.*, 1991; Rodrigues *et al.*, 1995). The location of the sequences of the primers were derived as shown in Fig. 1.

The oligonucleotides were made with a DNA synthesizer (Oligo 1000 DNA Synthesizer, Beckman, Mississauga, Ont) using the supplier's reagents and protocols.

b) Restriction enzyme digestion of the PCR amplicon. Restriction enzyme digestion analysis was performed to verify if the PCR amplicons consisted of the nisin structural gene sequence. The restriction enzyme digestion sites of published nisin gene sequence (Kuipers *et al.*, 1993) were analyzed using PC/Gene[®] computer program (IntelliGenetics, Inc.). The PCR amplicon of the nisin gene contains a single *SacI* restriction site (Fig. 1). Therefore, the PCR amplicons were digested with *SacI* using the buffer and protocol recommended by the manufacturer (Boehriner Mannheim).

c) Sequencing of the PCR amplicon. Both strands of the PCR amplicon were sequenced using primers 2 and 3, respectively, which were the same as those used for PCR amplification of the nisin gene. Amplicon purification, sequencing and sequence analysis were performed using the methods described above for 16S RNA gene sequencing.

Fig. 1. Restriction map of nisin gene cluster and the location of the primers used for PCR analysis and sequencing. The primers are shown as numbered arrows below the nisin structural gene. Lines connecting primers represent the amplified fragments with sizes given in base pairs.



2.7 Location of the nisin Z gene

2.7.1 Curing of plasmids

Plasmids were cured by growing the isolates in MRS broth containing acridine orange ($3 \mu\text{g mL}^{-1}$) at 40°C for 6 to 10 d until a plasmid-less strain was obtained (Hoover *et al.*, 1988). The plasmid analysis method described above were used to determine whether the plasmids were cured. The antil-listerial activity of the plasmid-less derivative was determined by using the well-diffusion method.

2.7.2 Hybridization of nisin Z gene probe with genomic DNA

To confirm that the bacteriocin gene was located on the chromosome, a nisin Z gene probe was used to hybridize with the chromosomal DNA of bean-sprout isolates after the gene was identified.

Chromosomal DNA was digested with *Sma*I and separated by PFGE. DNA from the agarose gels was transferred and fixed to nylon membranes using the methods described by Ng *et al.* (1993). Briefly, the DNA from the agarose gels was transferred by capillary action to Hybond™-N nylon membranes (Amersham Corp., Oakville, Ont) with 0.4 N NaOH as transfer solution. The transferred DNA was fixed by using GS Gene Linker™ UV Chamber (Bio-Rad) and then used for hybridization. A Random Primed DNA-Labeling Kit (Boehringer Mannheim) was used with [^{32}P]dCTP to generate a radiolabelled nisin structural gene probe. Unincorporated nucleotides were removed by using a Sephadex G-50 spin column (Boehringer Mannheim). Hybridizations were carried out with low stringency at 42°C and high stringency at 68°C in a solution of 6 x SSC (1 x SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50% formamide, 1% Denhardt solution, and $50 \mu\text{g mL}^{-1}$ of salmon

sperm DNA (Sigma). Hybridization stringency and protocol were based on that described by Sambrook *et al.*(1989b).

2.8 Copy number of nisin Z structural gene

The copy number of the nisin Z gene was confirmed using Southern blotting. Genomic DNA of the isolates was digested with *EcoRI* (Boehringer Mannheim). DNA fragments were resolved by electrophoresis in 0.6% agarose gels at 1 V cm⁻¹ for 18 h, then subjected to Southern hybridization with the two different nisin structural gene probes (311 and 120 bp), using the conditions described above.

2.9 Anti-listerial activity of bean-sprout isolates

2.9.1 Co-culture test in medium broth

Bean sprout isolate 80 was cultured with *L. monocytogenes* Scott A in MRS buffered with 1% β-glycerophosphate. Erlenmeyer flasks (250 mL) were filled with 100 mL of buffered MRS and inoculated with appropriate dilutions of an overnight culture of each organism. β-glycerophosphate was added to avoid rapid decreases in pH during the growth of *L. lactis* subsp. *lactis*. Target and inhibitor strains were inoculated to obtain ratios of 10³:10⁵ and 10³:10⁷ cells mL⁻¹, respectively, then incubated at 4 and 10°C for 24 d. At regular intervals, serial dilutions were plated onto both polymyxin acriflavine LiCl ceftazidime esculin mannitol (PALCAM) *Listeria* selective agar (BDH, Toronto, Ontario) and LiCl-chloride-phenylethanol-moxalactam (LPM) agar (Sigma) for the enumeration of *L. monocytogenes*, and on TSA-YE plates for the enumeration of *L. lactis*. Plates were read after incubation at 30°C for 48 h. Preliminary studies showed that *L. lactis* subsp. *lactis* did not grow on LPM or PALCAM agar.

2.9.2 Anti-listerial spectrum

The anti-listerial spectrum was tested using the well-diffusion and deferred-antagonism methods (Hoover and Harlander, 1993). Overnight culture (5 μ L) of bean-sprout isolate was added on TSA-YE agar and incubated for 18 h at 30°C. Then 7 mL of indicator-seeded molten TSA-YE agar (0.75%) was overlaid on top of the plate. Inhibition was measured after 18 h incubation. Different strains of *L. monocytogenes* and different species of *Listeria* were used as indicators.

2.9.3 Inoculation of bean sprout isolate onto fresh-cut-ready-to-eat vegetables

Bean-sprout isolate 80 was tested for its anti-listerial activity in fresh-cut-ready-to-eat Caesar salad, which is the most common retail FCV (J.M. Farber, personal communication). In order to reflect the real situation in food processing, beside strain *L. monocytogenes* Scott A, four other *L. monocytogenes* isolates from vegetables (HPB 2, 534, 540 and 845, Table 1), were used as an inoculum “pool” in the FCV tests. In pre-experimental trials, it was found that while three different inoculating levels, i.e. 10^3 , 10^6 and 10^8 cells g^{-1} , of bean-sprout isolate 80 had similar anti-listerial effects, the 10^8 cells g^{-1} inoculum gave the most consistent results. Therefore, an inoculum of 10^8 cells g^{-1} were used for the salad experiments. In addition, a combination of both bean-sprout isolates and a bacteriocin producing strain of *E. faecium* was also tested for effectiveness as starter cultures.

Different batches of Caesar salad (net weight 175 g bag^{-1}) were purchased from a local supermarket in the time period of 6 monthes. The inoculum was directly injected into the salad bag using a 3 mL-syringe. After inoculation, the bag was shaken vigorously for 3 min to completely mix the inoculum with the salad. First, each bag was inoculated with 2

mL of inoculum so as to give a final concentration of *L. monocytogenes* of 100 cells g⁻¹ of vegetable. The inoculum consisted of a mixture of five strains as follows: HPB 2, a coleslaw isolate of serotype 4b; HPB 3, Scott A, serotype 4b; HPB 534, a beet isolate of serotype 1 ; HPB540, a green bean isolate of serotype 1; and HPB 845, a cabbage isolate of serotype 4. Then, peptone water (2 mL) containing bean-sprout isolate 80, *E. faecium* ATCC19434, or a combination of bean-sprout isolate 80 and *E. faecium* ATCC19434, was inoculated into the salads to achieve a final concentration of starter culture of 10⁸ cells g⁻¹ of vegetable. The inoculated and control salad samples were incubated at 7 or 10°C for up to 10 d. On days 0, 3, 7 and 10, samples (100 g) from duplicate bags were placed into a Stomacher™ bag and pummelled with 200 mL of peptone water. Then, 0.2 mL of the diluted samples was placed onto LPM, PALCAM and MRS agar plates. The total number of *L. monocytogenes* colonies on LPM and PALCAM agar plates and the total number of bacteria on MRS agar were counted after incubating the plates at 30°C for 48 h. The pH of the samples was measured with an Orion's Solid State pH meter (Model 610, Orion Research, Boston, Ma.). The headspace composition of the packaged salads was analyzed with a Varian gas chromatograph (Model 3300, Varian Canada, Inc., Toronto, Ont.) as previously described (Farber *et al.*, 1996). Salads were examined and confirmed not to contain any naturally occurring *Listeria* by using the HPB *L. monocytogenes* isolation method (MFHPB-30; Farber *et al.*, 1994).

2.10 Statistical analysis

All the experiments were repeated at the least two times. FCV Caesar salad experiments were repeated using different batches of vegetables. Results of salad

experiments were expressed as \log_{10} of the mean of four to six samples of two experiments. Statistical analysis was carried out using one- or two-way ANOVA on the \log_{10} transformed data, using GraphPad Prism statistics software package version 2.00 (GraphPad Prism Software Inc., San Diego, CA). Significant differences between treatment groups were determined by using Tukey's multiple comparison test. Statistical significance was inferred at $P < 0.05$.

The dendrogram from PFGE banding patterns was generated using Molecular Analyst/PC Fingerprinting Software version 1 from Dice correlations and unweighted pair group method using arithmetic averages (UPGMA) clustering.

3. RESULTS

3.1 Isolation of and identification of bacteriocin producing strains

Among a total of 76 isolates from bean sprouts, 34 produced antimicrobial compounds which inhibited the indicator organism, *L. monocytogenes* Scott A. The inhibition zones were approximately 22 mm in diameter, as tested by the well-diffusion method (Fig. 2). Ten of the isolates which exhibited the biggest inhibition zones were selected for further characterization.

3.2 Speciation of bacteriocin producing strains

3.2.1 Morphology

The isolates were gram-positive, non-spore-forming cocci. Under the electron microscope, they appeared as cocci and had no flagella (Fig. 3).

3.2.2 Biological tests

The biochemical characteristics of the bean-sprout isolates tested by the VITEK can be seen in Table 2. All of the isolates fermented cellobiose, dextrose, esculin, salicin, sucrose, trehalose, xylose and ribose, deaminated arginine and grew in the presence of tetrazolium red, as well as 10 and 40% bile. Most isolates (9/10) did not ferment arabinose or raffinose (8/10), and did not grow (8/10) in 6% NaCl. In addition, 8 of 10 strains fermented lactose, while half of them fermented mannitol. The isolates were presumptively identified as enterococci by the VITEK test, i.e., isolates 80, 72, 75, 77, 54, 52, 47, 42 and 40 as *E. faecium* (probability >99%), and isolate 32 as *Enterococcus gallinarum* (60% probability) and *E. faecium* (40% probability).

3.2.3 DNA hybridization

In order to confirm the identification of bean-sprout isolates tested by the VITEK system, DNA hybridization experiments were performed. None of the ten isolates hybridized with the *Enterococcus* specific gene probe (Table 3), indicating that these strains were not enterococci.

3.2.4 Growth characteristics

Additional biochemical tests were performed to identify the bean-sprout isolates after the DNA hybridization results were found to be different from the VITEK results. The isolates were not able to grow at 45°C, but grew in the presence of 4% NaCl at 30°C. One of the isolates, isolate 80, was subjected to a low temperature growth test. The organism survived at 3 to 4.5°C for at least 20 d, grew at 4°C, and produced anti-listerial material at 5°C (data not shown).

On the basis of the biochemical tests and growth characteristics, the isolates were tentatively identified as *L. lactis* subsp. *lactis*.

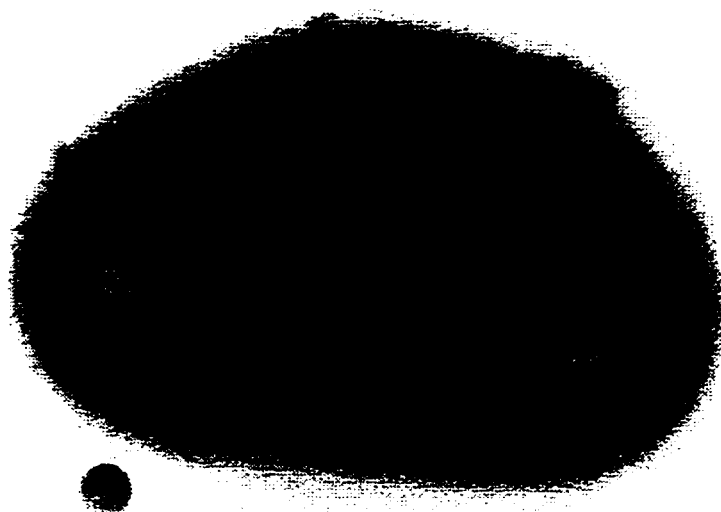
3.2.5 Ribotyping

To further confirm the identification of the bean-sprout isolates, two isolates (80 and 32) with the biggest inhibition zones were selected for ribotyping. Isolate 32 and 80 both produced the same RiboPrint® pattern, which was identical to one in the database for *L. lactis* subsp. *lactis* (data not shown).

Fig. 2. Inhibition of *L. monocytogenes* by *L. lactis* subsp. *lactis* isolated from bean sprouts as measured by well-diffusion assay. The indicator organism was *L. monocytogenes* Scott A. The diameter of the inhibition zone was 22 mm.



Fig. 3. Transmission electron micrograph of bean-sprout isolate 80. The isolates were gram-positive (by Gram-stain), non-flagellated, and non-spore forming cocci.



— 0.6 μM

Table 2. Identification of bean-sprout isolates by the VITEK test

	Isolate no.									
	32	80	77	75	72	54	52	47	42	40
Arabinose	-	-	-	-	+	-	-	-	-	-
Bacitracin	+	+	+	+	+	+	+	+	+	+
Catalase	-	-	-	-	-	-	-	-	-	-
Cellobiose	+	+	+	+	+	+	+	+	+	+
Dextrose	+	+	+	+	+	+	+	+	+	+
Esculin	+	+	+	+	+	+	+	+	+	+
Hemicellulase	+	+	+	+	+	+	+	+	+	+
Inulin	-	-	-	-	-	-	-	-	-	-
Lactose	+	+	+	-	+	+	-	+	+	+
Mannitol	+	+	-	-	-	+	-	+	+	-
Melezitose	-	-	-	-	-	-	-	-	-	-
Melibiose	+	-	-	-	-	-	-	-	-	-
Novobiocin	+	+	+	+	+	+	+	+	+	+
Optochin+	+	+	+	+	+	+	+	+	+	
Peptone base	+	+	+	+	+	+	+	+	+	+
Pullulan	-	-	-	-	-	-	-	-	-	-
Pyruvate	-	-	-	-	-	-	-	-	-	-
Raffinose	+	-	-	-	+	-	-	-	-	-
Salicin	+	+	+	+	+	+	+	+	+	+
Sorbitol	-	-	-	-	-	-	-	-	-	-
Sucrose	+	+	+	+	+	+	+	+	+	+
β -Hemolytic	-	-	-	-	-	-	-	-	-	-
Tetrazolium red	+	+	+	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+	+	+	+
Urea	-	-	-	-	-	-	-	-	-	-
10% Bile	+	+	+	+	+	+	+	+	+	+
40% Bile	+	+	+	+	+	+	+	+	+	+
Xylose	+	+	+	+	+	+	+	+	+	+
6%NaCl	+	-	-	-	-	+	-	-	-	-
Ribose	+	+	+	+	+	+	+	+	+	+
Arginine	+	+	+	+	+	+	+	+	+	+

Table 3. Identification of bean-sprout isolates using the *Enterococcus* specific DNA probe (AccuProbe™)

Tested strains	PLU ^a	Hybridization ^b
Bean-sprout isolate 32	154±10	negative
Bean-sprout isolate 40	95±9	"
Bean-sprout isolate 42	100±12	"
Bean-sprout isolate 47	102±11	"
Bean-sprout isolate 52	128±13	"
Bean-sprout isolate 54	77±10	"
Bean-sprout isolate 72	191±9	"
Bean-sprout isolate 75	101±10	"
Bean-sprout isolate 77	191±10	"
Bean-sprout isolate 80	136±13	"
<i>Enterococcus faecium</i> ATCC 19434 ^c	10,025±30	positive
<i>Enterococcus gallinarum</i> ATCC 35038 ^c	10,470±20	positive
<i>Streptococcus bovis</i> ^d	99±10	negative

^a PLU-photometric light unit; values are the means±SE of two experiments.

^b Cut-off values: positive >1,500 PLU, negative <600 PLU.

^c Positive control.

^d Negative control.

3.2.6 16S rRNA gene sequencing

Based on the alignment with 16S rRNA sequences in GeneBank database (Blastn 1.4.8 MP, built at 08:41:09 Oct 19, 1995), the majority (1538 bp) of the 16S rRNA gene sequence of isolate 80 was obtained. This identified sequence was 99.7% homologous to that of *L. lactis* subsp. *lactis* (1548 bp) as reported by Chiaruttini and Milet (1993) (Fig. 4).

3.3 **Comparison of the bacteriocin producing bean-sprout isolates**

3.3.1 Plasmid profile

Both the modified Birnboim and Doly method (1979) and the Wizard™ Minipreps DNA purification methods gave similar results. There were four different plasmid profiles: (I) isolate 80 and 32 contained a 25 Kb and a 9 Kb plasmid; (II) isolates 40, 42, 47,52, 54 and 74 contained a 45 Kb and a 25 Kb plasmids; (III) isolate 77 contained a 45 Kb plasmid; and (IV) isolate 72 contained a 25 Kb, 10 Kb, 4.5 Kb and 2.8 Kb plasmids (Fig. 5).

3.3.2 Antibiogram

The isolates showed identical antibiograms (Table 4). All isolates, including strain 32j, the plasmid-free derivative of isolate 32, were sensitive to ampicillin, cefotaxime, chloramphenicol, gentamicin, tetracycline, trimethoprim-sulfamethoxazole, sulfisoxazole, cephalothin and vancomycin; resistant to nalidixic acid, streptomycin and polymyxin, and showed intermediate sensitivity to kanamycin.

Fig. 4. 16S rRNA gene alignment of bean-sprout isolate 80 and *L. lactis* subsp. *lactis* (LLL). The complete sequence of 16S rRNA gene of *L. lactis* subsp. *lactis* was 1548 bp, as reported by Chiaruttini and Milet (1993), accession no. x64887. The majority (1538 bp) of the 16S rRNA gene sequence of isolate 80 was sequenced. Base number 304 was not readabale and is referred to as N in the figure. The identified sequence was 99.7% homologous to that of *L. lactis* subsp. *lactis* as reported by Chiaruttini and Milet (1993).

```

#80 - AGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCT-40
      |||||||||||||||||||||||||||||||||||||||
LLL - TTTATTTGAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCT-50

#80 - AATACATGCAAGTTGAGCGCTGAAGGTTGGTACTTGTACCGACTGGATGA-90
      |||||||||||||||||||||||||||||||||||||||
LLL - AATACATGCAAGTTGAGCGCTGAAGGTTGGTACTTGTACCGACTGGATGA-100

#80 - GCAGCGAACGGGTGAGTAACGCGTGGGGAATCTGCCTTTGAGCGGGGGAC-140
      |||||||||||||||||||||||||||||||||||||||
LLL - GCAGCGAACGGGTGAGTAACGCGTGGGGAATCTGCCTTTGAGCGGGGGAC-150

#80 - AACATTTGAAACGAATGCTAATACCGCATAAAAACTTTAAACACAAGTT-190
      |||||||||||||||||||||||||||||||||||||||
LLL - AACATTTGAAACGAATGCTAATACCGCATAAAAACTTTAAACACAAGTT-200

#80 - TTAAGTTTGAAAGATGCAATTGCATCACTCAAAGATGATCCCGCGTTGTA-240
      |||||||||||||||||||||||||||||||||||||||
LLL - TTAAGTTTGAAAGATGCAATTGCATCACTCAAAGATGATCCCGCGTTGTA-250

#80 - TTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCGATGATACATAGCCGA-290
      |||||||||||||||||||||||||||||||||||||||
LLL - TTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCGATGATACATAGCCGA-300

#80 - CCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGNCCCAAACCTCCT-340
      |||||||||||||||||||||||||||||||||||||||
LLL - CCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAACCTCCT-350

#80 - ACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGA-390
      |||||||||||||||||||||||||||||||||||||||
LLL - ACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGA-400

#80 - GCAACGCCCGCTGAGTGAAGAAGGTTTTTCGGATCGTAAAACTCTGTTGGT-440
      ||||||||.|||||||||||||||||||||||||||||
LLL - GCAACGCCCGCTGAGTGAAGAAGGTTTTTCGGATCGTAAAACTCTGTTGGT-450

#80 - AGAGAAGAACGTTGGTGAGAGTGAAAGCTCATCAAGTGACGGTAACTAC-490
      |||||||||||||||||||||||||||||||||||||||
LLL - AGAGAAGAACGTTGGTGAGAGTGAAAGCTCATCAAGTGACGGTAACTAC-500

#80 - CCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGT-540
      |||||||||||||||||||||||||||||||||||||||
LLL - CCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGT-550

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#80 -CCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGTGGTTTA-590
    |||
LLL -CCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGTGGTTTA-600

#80 -TTAAGTCTGGTGTAAAAGGCAGTGGCTCAACCATTGTATGCATTGGAAAC-640
    |||
LLL -TTAAGTCTGGTGTAAAAGGCAGTGGCTCAACCATTGTATGCATTGGAAAC-650

#80 -TGGTAGACTTGAGTGCAGGAGAGGAGAGTGGAAATTCATGTGTAGCGGTG-690
    |||
L.L.L -TGGTAGACTTGAGTGCAGGAGAGGAGAGTGGAAATTCATGTGTAGCGGTG-700

#80 -AAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGCCTCTCTGGC-740
    |||
LLL -AAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGC-750

#80 -CTGTAACTGACACTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGCT-790
    |||
LLL -CTGTAACTGACACTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGAT-800

#80 -ACCCTGGTAGTCCACGCCGTAACGATGAGTGCTAGATGTAGGGAGCTAT-840
    |||
LLL -ACCCTGGTAGTCCACGCCGTAACGATGAGTGCTAGATGTAGGGAGCTAT-850

#80 -AAGTTCTCTGTATCGCAGCTAACGCAATAAGCACTCCGCCTGGGGAGTAC-890
    |||
LLL -AAGTTCTCTGTATCGCAGCTAACGCAATAAGCACTCCGCCTGGGGAGTAC-900

#80 -GACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGT-940
    |||
LLL -GACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGT-950

#80 -GGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTG-990
    |||
LLL -GGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTG-1000

#80 -ACATACTCGTGCTATTCCCTAGAGATAGGAAGTTCCTTCGGGACACGGGAT-1040
    |||
LLL -ACATACTCGTGCTATTCCCTAGAGATAGGAAGTTCCTTCGGGACACGGGAT-1050

#80 -ACAGGTGGTGCATGGTTGTCGTGAGCTCGTGTGAGATGTTGGGTTAA-1090
    |||
LLL -ACAGGTGGTGCATGGTTGTCGTGAGCTCGTGTGAGATGTTGGGTTAA-1100

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#80 -GTCCCGCAACGAGCGCAACCCCTATTGTTAGTTGCCATCATTAAGTTGGG-1140
      |||||||||||||||||||||||||||||||||||||||||||||||||||
LLL -GTCCCGCAACGAGCGCAACCCCTATTGTTAGTTGCCATCATTAAGTTGGG-1150

#80 -CACTCTAACGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGT-1190
      |||||||||||||||||||||||||||||||||||||||||||||||||||
LLL -CACTCTAACGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGT-1200

#80 -CAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGAT-1240
      |||||||||||||||||||||||||||||||||||||||||||||||||||
LLL -CAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGAT-1250

#80 -GGTACAACGAGTCGCGAGACAGTGATGTTTAGCTAATCTCTTAAAACCAT-1290
      |||||||||||||||||||||||||||||||||||||||||||||||||||
LLL -GGTACAACGAGTCGCGAGACAGTGATGTTTAGCTAATCTCTTAAAACCAT-1300

#80 -TCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGC-1340
      |||||||||||||||||||||||||||||||||||||||||||||||||||
LLL -TCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGC-1350

#80 -TAGTAATCGCGGATCAGCACGCCGCGTGAATACGTTCCCGGGCCTTGTA-1390
      |||||||||||||||||||||||||||||||||||||||||||||||||||
LLL -TAGTAATCGCGGATCAGCACGCCGCGTGAATACGTTCCCGGGCCTTGTA-1400

#80 -CACACCGCCCGTCACACCACGGGAGTTGGGAGTACCCGAAGTAGGTTGCC-1440
      |||||||||||||||||||||||||||||||||||||||||||||||||||
LLL -CACACCGCCCGTCACACCACGGGAGTTGGGAGTACCCGAAGTAGGTTGCC-1450

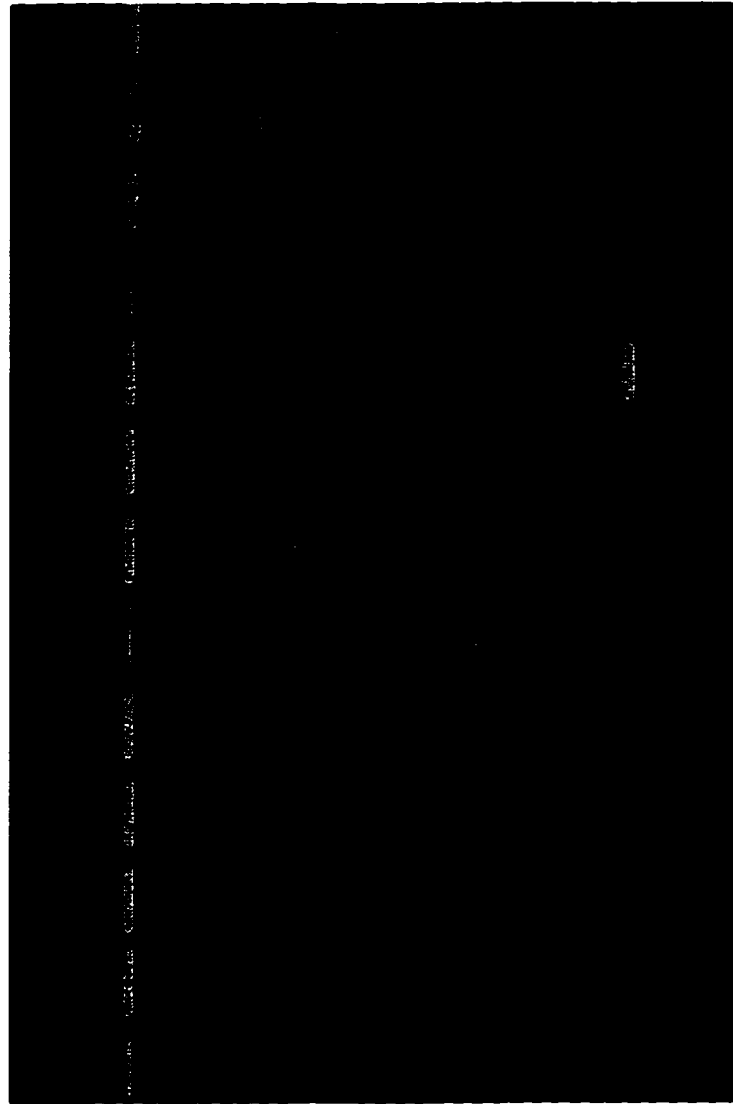
#80 -TAACCGCAAGGAGGGCGCTTCCTAAGGTAAGACCGATGACTGGGGTGAAG-1490
      |||||||||||||||||||||||||||||||||||||||||||||||||||
LLL -TAACCGCAAGGAGGGCGCTTCCTAAGGTAAGACCGATGACTGGGGTGAAG-1500

#80 -TCGTAACAAGGTAGCCGTATCGGAAGGTGCGGCTGGATCACCTCCTTA-1538
      |||||||||||||||||||||||||||||||||||||||||||||||||||
LLL -TCGTAACAAGGTAGCCGTATCGGAAGGTGCGGCTGGATCACCTCCTTT-1548

```

Fig. 5. Plasmid profiles of bean-sprout isolates. Four different plasmid profiles were obtained among bean-sprout isolates: (I) isolates 32 and 80 contained a 25 Kb and a 9 Kb plasmid; (II) isolates 40, 42, 47, 52, 54, 74 contained a 45Kb and 25 Kb plasmid; (III) isolate 77 contained a 45 Kb plasmid; and (IV) isolate 72 contained a 25 Kb, 10 Kb, 4.5 kb and 2.8 Kb plasmid. DNA size marker: m1: supercoiled DNA ladder (Gibco BRL); m2 and m3: plasmid RP1 (57.6 Kb) and plasmid R222 (90.9 Kb) (Mendez *et al.*, 1980). The 16 Kb bands were chromosomal DNA. The plasmid sizes were average sizes determined by isolating and analysing the plasmids at least three times.

Strain # : 32 40 42 47 52 m1 54 75 72 77 80 m2 m3



plasmid
size(Kb)

45 —
25 —

10 —
9 —

4.5 —
2.8 —

Marker:m1-3
(Kb)

91 —
58 —

16 —
12 —
10 —
8 —

5 —
4 —
3 —
2 —

Table 4. Antibigrams^a of various bean-sprout isolates

Antibiotics	Susceptibility ^b of beansprout isolates									
	32	32j	40	42	52	54	72	75	77	80
Ampicillin (10 µg)	S	S	S	S	S	S	S	S	S	S
Cefetaxime (30 µg)	S	S	S	S	S	S	S	S	S	S
Chloramphenicol (30 µg)	S	S	S	S	S	S	S	S	S	S
Gentamicin (10 µg)	S	S	S	S	S	S	S	S	S	S
Kanamycin (30 µg)	I	I	I	I	I	I	I	I	I	I
Polymyxin B (300 Units)	R	R	R	R	R	R	R	R	R	R
Streptomycin (10 µg)	R	R	R	R	R	R	R	R	R	R
Trimethoprim (1.25 µg)- Sulfamethoxazole (23.75 µg)	S	S	S	S	S	S	S	S	S	S
Tetracycline (30 µg)	S	S	S	S	S	S	S	S	S	S
Sulfisoxazole (300 µg)	S	S	S	S	S	S	S	S	S	S
Cephalothin (30 µg)	S	S	S	S	S	S	S	S	S	S
Nalidixic acid (30 µg)	R	R	R	R	R	R	R	R	R	R
Vancomycin (30 µg)	S	S	S	S	S	S	S	S	S	S

^a Determined using Dispens-O-Disc™ Susceptibility Test System (Difco), a disk-diffusion test system, using the supplier's protocol, interpretive standards and quality control limits.

^b S, susceptible; I, intermediate susceptibility; R, resistant.

3.3.3 PFGE typing

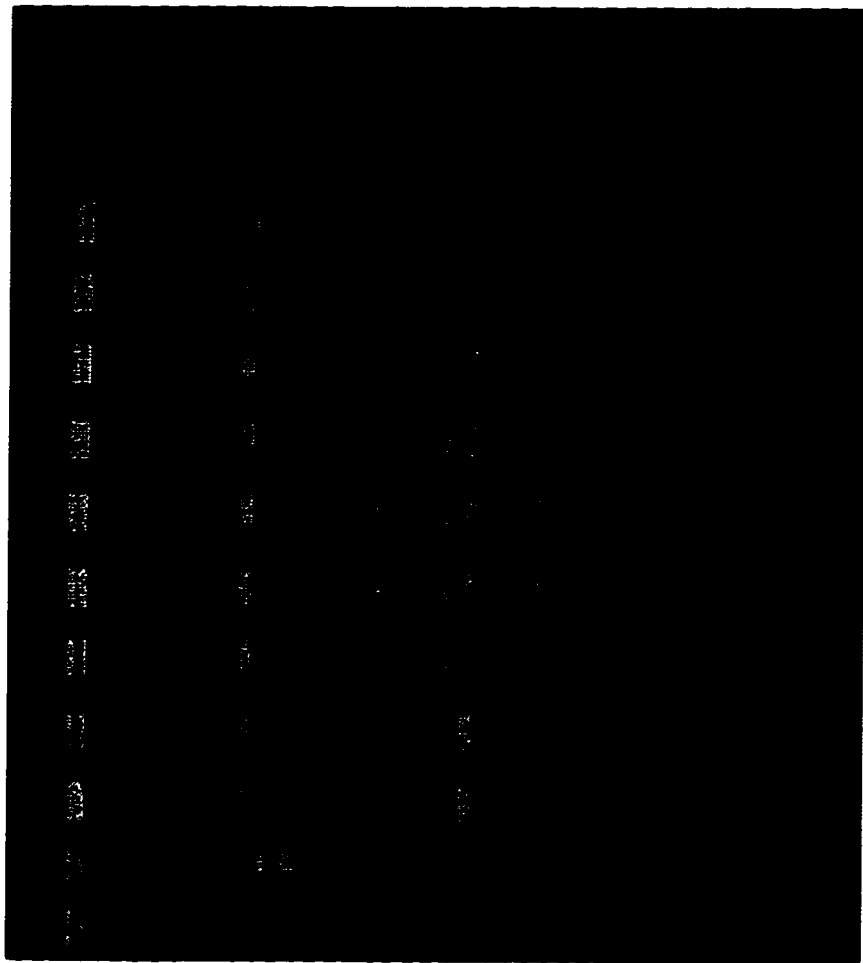
The bean-sprout isolates could be divided into four groups of one or more band difference in PFGE patterns. Isolates 40, 42, 47, 52, 54, 75 and 77 were identical (type IV); isolate 80 (type III) was similar to this group except for one missing band (50 Kb); isolates 32 and 72 had a unique pattern (type II and I); and all isolates were different from the sauerkraut isolates (Fig. 6). In the dendrogram (Fig. 7), isolate 72 was different from all other bean-sprout isolates with only 22.8% similarity . Isolate 32 had 72% similarity to other bean-sprout isolates except isolate 72. Isolate 40, 42, 47, 52, 54, 75 and 77 fell into the same PFGE type with a similarity index of 100%; isolate 80 had 95% similarity with this group of isolates. The sauerkraut isolates were markedly different from all bean-sprout isolates (except isolate 72) with only 22.8% similarity, and different from isolate 72 with 42% similarity .

3.3.4 Summary of bean-sprout isolate comparison

Comprehensive analysis of the results of the bean-sprout isolate typing using PFGE typing, plasmid profiles and antibiograms placed the ten isolates into five subtypes (Table 5). Isolates 32, 72, 77 and 80 each had unique overall subtyping profiles. Isolate 72 had a unique PFGE pattern and plasmid profile, while isolates 32 and 80 shared a unique plasmid profile but could be differentiated by PFGE typing. Isolates 40, 42, 47, 52, 54, and 75 shared a common subtype with identical plasmid and PFGE patterns. Isolate 77 had the same PFGE banding profile as the latter group, but could be differentiated by plasmid typing.

Fig. 6. PFGE patterns from *Sma*I digested genomic DNA of bean-sprout isolates. Lanes 1 and 14: λ DNA ladder (Boehringer Mannheim); Lanes 2-11: bean-sprout isolates; NCK400 and NCK402: *L. lactis* subsp. *lactis* nisin positive and nisin negative sauerkraut isolates, respectively.

Strain #: 72 32 80 77 54 52 40 42 47 75
Patterns: I II III IV IV IV IV IV IV IV V
 NCK400 NCK402
 λladder λladder



Kb
 — 291.0
 — 242.5
 — 194.0
 — 145.5
 — 97.0
 — 48.5

Fig. 7. Dendrogram of bean-sprout isolates generated from PFGE banding patterns.

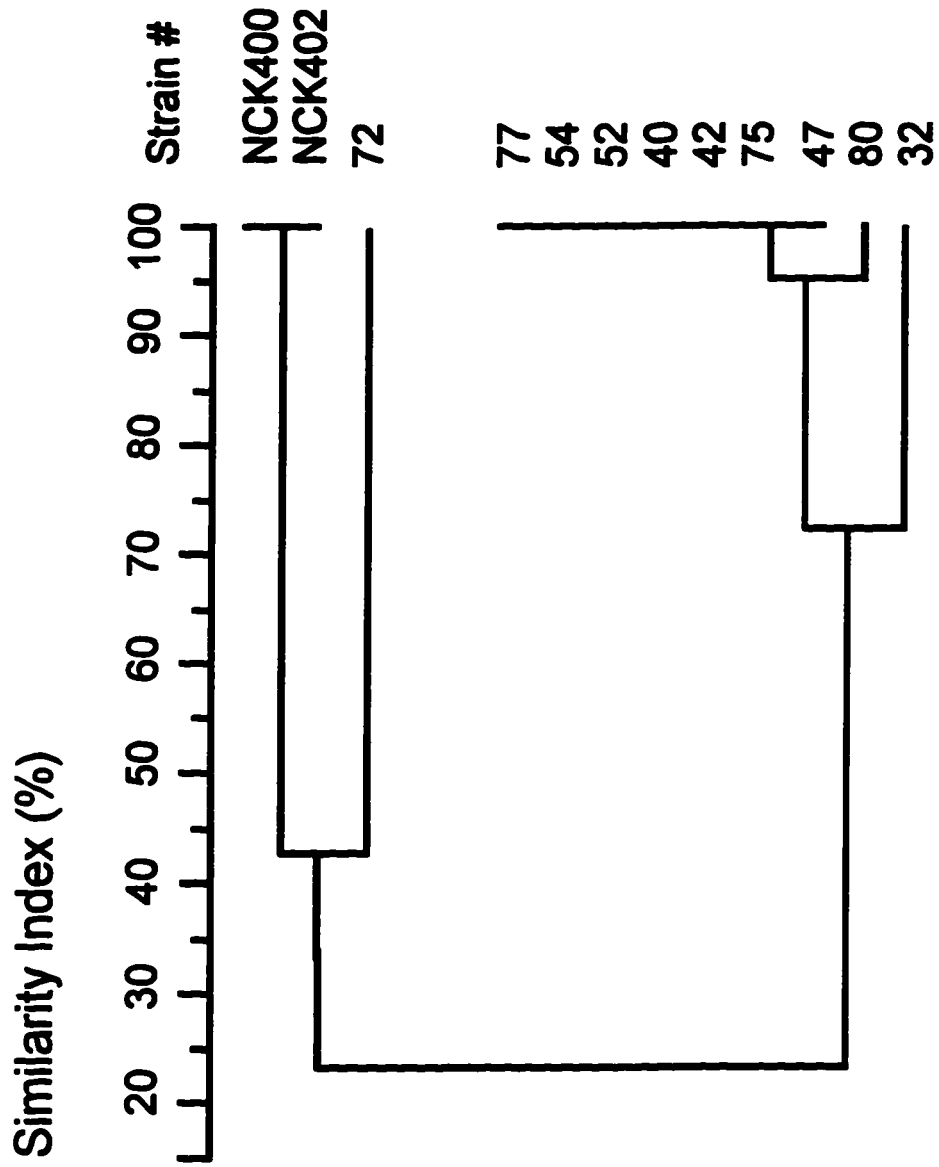


Table 5. Summary of phenotypic and molecular typing of bean-sprout isolates

Isolate no.	Plasmid profiles	PFGE patterns	Antibiogram	Comprehensive subtype ^a
32	I	I	I	I
40	II	IV	I	II
42	II	IV	I	II
47	II	IV	I	II
52	II	IV	I	II
54	II	IV	I	II
72	III	II	I	III
75	II	IV	I	II
77	IV	IV	I	IV
80	I	III	I	V
D ^b	0.64	0.53	0	0.66

^a Arbitrary group designation based on overall profiles from three typing methods.

^b Numerical index of discrimination, $D = 1 - [1/N(N-1)] \sum_{j=1}^s n_j(n_j-1)$ (Hunter and Gaston, 1988)

3.4 Characterization of the anti-listerial compounds

3.4.1 pH effects

The supernatant of the isolates retained their anti-listerial effects after being neutralized to pH 7 (Table 6), implying that the anti-listerial effects were not due to acid in the supernatant.

3.4.2 Phage effects

In the flip-gel streak test, isolate 80 had strong inhibitory effects against *L. monocytogenes* Scott A, indicating that the anti-listerial effects were not due to a bacteriophage (Fig. 8).

3.4.3 Heat resistance and enzyme profiles

The anti-listerial compound(s) were heat resistant and proteinaceous, being inactivated by proteinase K, protease, trypsin, α -chymotrypsin, pepsin and papain (Table 7), indicating that the anti-listerial compound(s) were bacteriocin-like. The bean-sprout isolates had similar heat resistance and enzyme profiles as that of sauerkraut isolate NCK400, a nisin producing strain of *L. lactis* subsp. *lactis* (Table 7). In addition, the activity was not abolished by catalase, indicating that the anti-listerial effects were not due to H₂O₂.

3.4.4 Bacteriocin immunity

Isolates 32 and 80 were resistant to the supernatant of *L. lactis* subsp. *lactis* NCK400 which produced nisin, and the supernatants of isolate 32 and 80 did not inhibit NCK400, indicating that the bean-sprout isolates had immunity against nisin (Table 8).

Table 6. The effect of pH neutralization on the anti-listerial activity of bean-sprout isolate supernatants

Strain no.	Inhibition zone (mm) ^a	
	Unneutralized supernatant ^b	Neutralized supernatant ^c
47	22.0±0.2	19.0±0.2
72	23.0±0.1	22.0±0.1
80	22.0±0.2	20.0±0.1

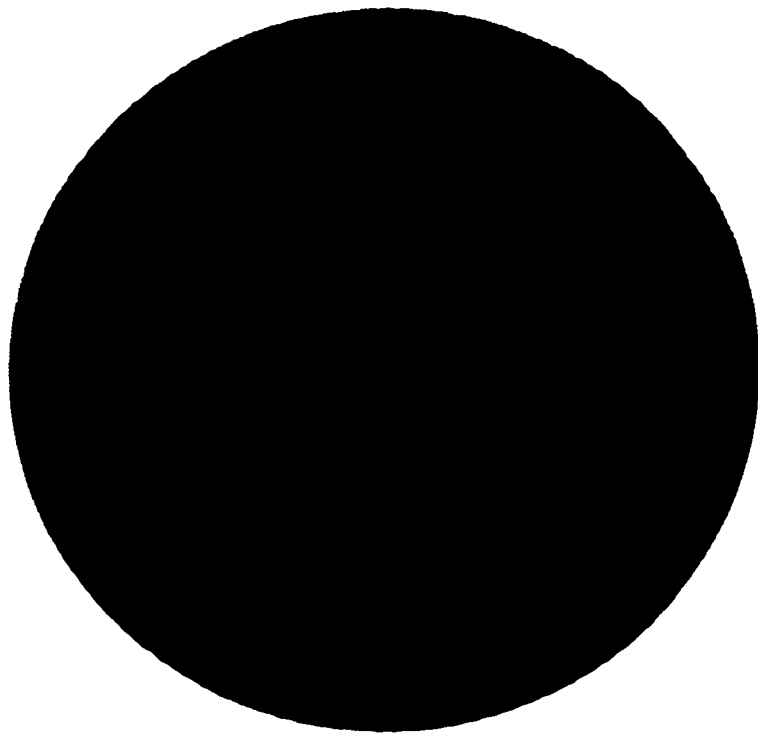
^a Well-diffusion method. Values are the means±SE of four samples from two experiments

^b pH 4.5.

^c Neutralized with 1 N NaOH to a pH value of 7.0.

Fig. 8. Flip-gel streak test. The growth of *L. monocytogenes* strain Scott A was inhibited along the streak of bean-sprout isolate 80.

Isolate 80



Scott A

Table 7. The effect of various enzymes and heat on the anti-listerial activity of bean-sprout isolate culture supernatants

Treatment	Bean-sprout isolates 32 and 80	NCK400 ^a
Proteinase K	+ ^b	+
Protease	+	+
Trypsin	+	+
Chymotrypsin	+	+
Pepsin	+	+
Papain	+	+
Catalase	- ^c	-
Heat (60°C, 30 min)	-	-
Heat (100°C, 10 min)	-	-
Autoclave (121°C, 15 psi, 15 min)	-	-

^a Sauerkraut isolate, nisin producing *L. lactis* subsp. *lactis*.

^b +; signifies no anti-listerial activity.

^c -; signifies anti-listerial activity.

Table 8. Immunity of bean-sprout isolates against nisin producing *L. lactis* subsp. *lactis*

NCK400

Indicator Strains		Inhibition zone (mm) ^a
Bean-sprout isolates	32	0
"	32j	0
"	40	0
"	42	0
"	47	0
"	52	0
"	54	0
"	75	0
"	72	0
"	77	0
"	80	0
<i>L. monocytogenes</i>	Scott A	15±0.1

^a Stabbing method

3.4.5 PCR amplification and sequencing of the nisin gene

PCR was performed using the genomic DNA of *L. lactis* subsp. *lactis* NCK400 and NCK402 as a positive and negative control, respectively, to identify if the nisin gene existed in the genomic DNA of isolates 80 and 32. Reactions carried out using primers 1 and 2 and primers 2 and 3, would amplify a 184 and 311 bp fragment, respectively, in those strains containing the nisin structural gene. DNA from isolates 32 and 80 generated amplicons of these sizes (Fig. 9), as did the eight other isolates (data not shown). Digestion of the 184 bp amplicon with *SacI* generated the expected fragments of 84 and 98 bp (Fig. 10). Primers 2 and 3 were subsequently employed in sequencing of the 311 bp fragments from isolates 80 and 32. The resulting sequences were 100% identical to the nisin Z structural gene, *nisZ* (Fig. 11).

3.5 Nisin gene location

3.5.1 Bacteriocin producing activity of the plasmid-free strain

Isolate 32j, a plasmid-cured strain derived from isolate 32 (Fig. 12) continued to produce bacteriocin, indicating that the nisin gene is probably located on the chromosome.

3.5.2 Hybridization of *nisZ* gene probe with genomic DNA of bean-sprout isolates

A PCR amplicon probe (311 bp), containing the whole *nisZ* gene, hybridized with *SmaI* digested chromosomal DNA fragments of bean-sprout isolates in Southern blotting analysis, indicating that the nisin gene was located on the chromosome (Fig. 13). Two fragments of genomic DNA from isolates 52 and 75 hybridized with the *nisZ* probe. The characteristics of these fragments need further investigation.

Fig. 9. Amplification of the *nisZ* gene of various bean-sprout isolates. By using a pair of nisin structural gene specific primers, the expected size, i.e., a 184 bp DNA fragment was amplified. NCK402 and NCK400 were nisin negative and positive strains of *L. lactis* subsp. *lactis*, respectively. M: DNA ladder marker 123 (size range: 123 bp and up; GIBCO BRL).

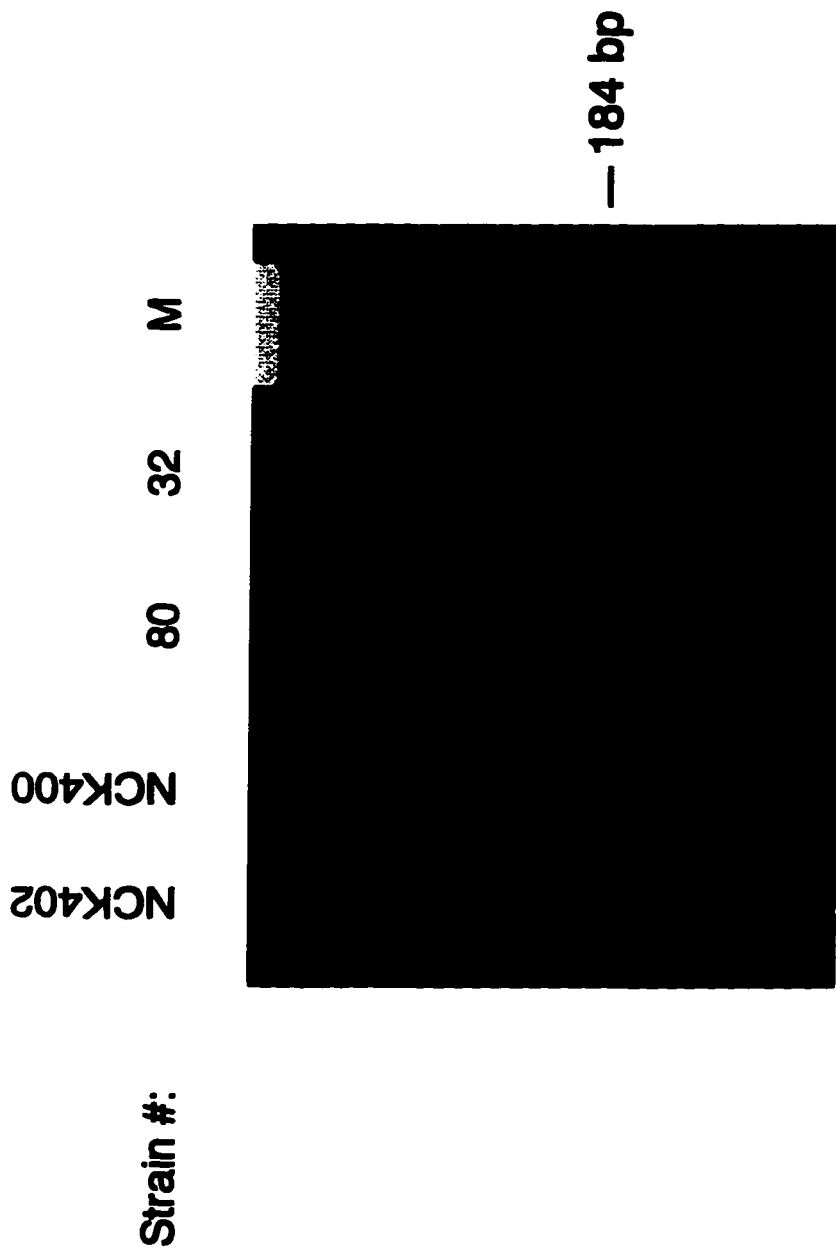


Fig. 10. Restriction analysis of PCR amplicons of the nisin gene from bean-sprout isolates. Amplicons in lanes 1, 3, 4 and 5 were digested with *SacI*, while that in lane 2 was undigested. Digestion of the nisin structural gene amplicon generated expected fragments (84 and 98 bp), which were visualized as one band in 1% agarose gel. NCK400 is a nisin positive strain of *L. lactis* subsp. *lactis*. M: DNA ladder marker 123 (size range: 123 bp and up, GIBCO BRL).

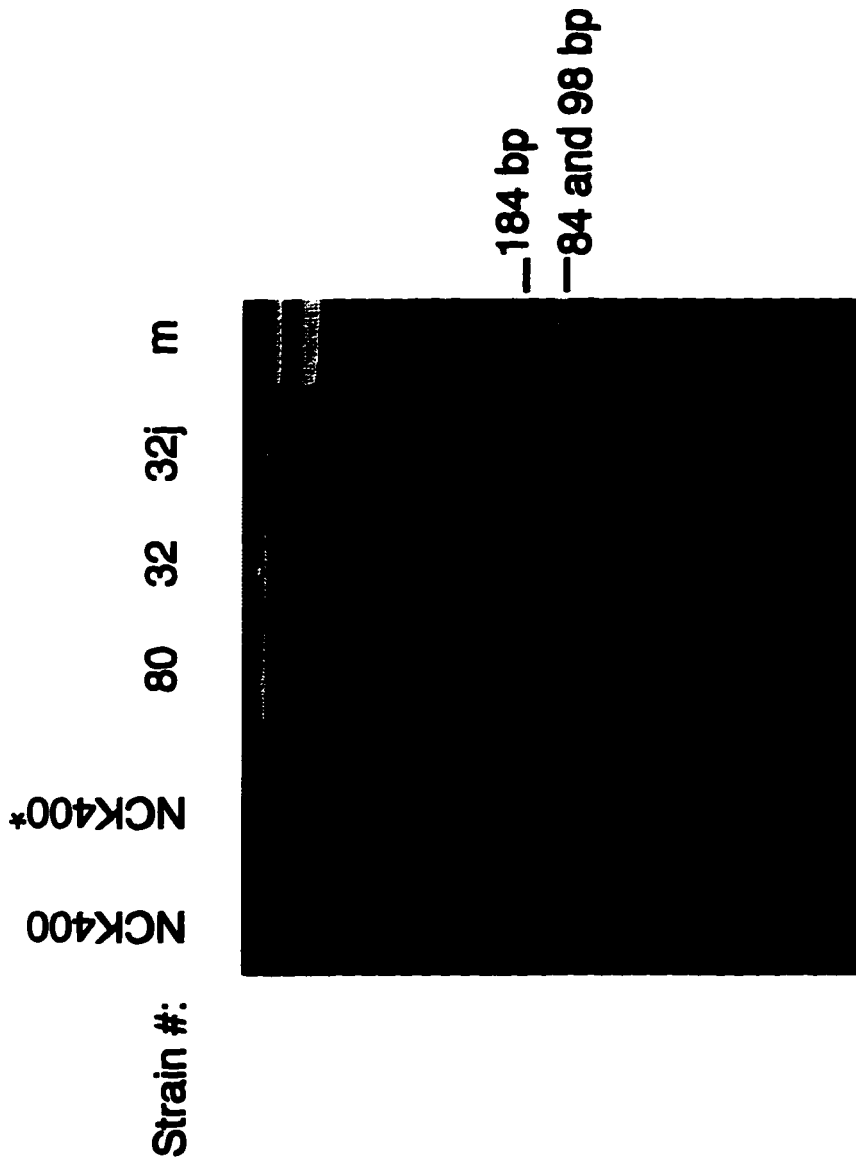


Fig. 11. Alignment of the DNA sequence of the nisin gene. PCR amplicon amplified with primers 2 and 3 from bean sprout isolates 32 and 80 was 100% homologous to the sequence of *nisZ*, the structural gene of nisin Z, as reported by Mulders *et al.* (1991), accession no. X61144.

Amplicon GTTTGTAGATACAATGATTTTCGTTCGAAGGAACTACAAAATAA
nis Z GTTTGTAGATACAATGATTTTCGTTCGAAGGAACTACAAAATA A

start

Amplicon ATTATAAGGAGGCACTCAAAAATGAGTACAAAAGATTTTAACTTG
nis Z ATTATAAGGAGGCACTCAAAAATGAGTACAAAAGATTTTAACTTG

Amplicon GATTTGGTATCTGTTTCGAAGAAAGATTCAGGTGCATCACCACG
nis Z GATTTGGTATCTGTTTCGAAGAAAGATTCAGGTGCATCACCACG

Amplicon CATTACAAGTATTTTCGCTATGTACACCCGTTGTAAAACAGGAGC
nis Z CATTACAAGTATTTTCGCTATGTACACCCGTTGTAAAACAGGAGC

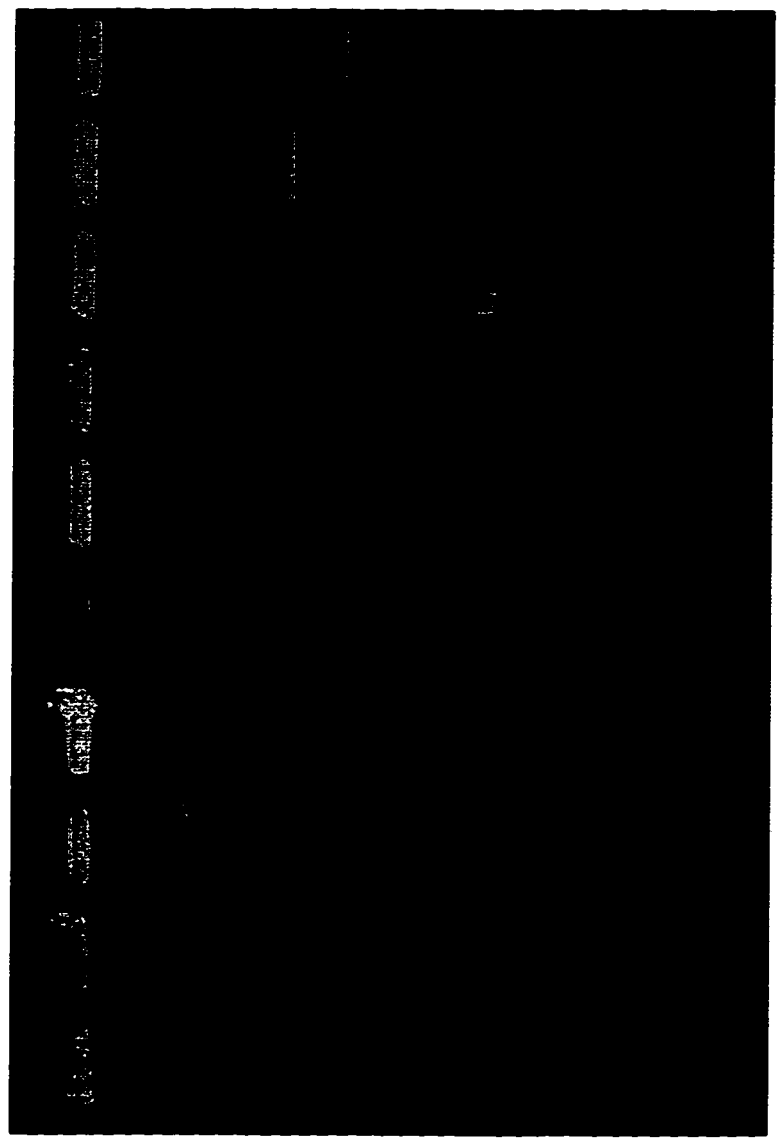
Amplicon TCTGATGGGTTGTAACATGAAAACAGCAACTTGTAATTGTAGTATT
nis Z TCTGATGGGTTGTAACATGAAAACAGCAACTTGTAATTGTAGTATT

stop

Amplicon CACGTAAGCAAATAACCAAATCAAAGGATAGTATTTTGTAGTTC
nis Z CACGTAAGCAAATAACCAAATCAAAGGATAGTATTTTGTAGTTC

Fig. 12. Curing of plasmids from bean-sprout isolates. The strains 80g3 and 32e, derivatives of isolates 80 and 32, respectively, were cured of their 25 Kb plasmids. Strains 32j1, 32j2 and 32j3 were plasmid-free derivatives of isolate 32. DNA size marker: M1: supercoiled DNA ladder (Gibco BRL); M2-4: plasmid RA1 (130.3 Kb), plasmid RP1 (57.6 Kb), and plasmid R222 (90.9 Kb)(Mendez *et al.*, 1980). All these cured strains retained their anti-listerial activity.

Strain #: 32e 32j1 32j2 32j3 m1 80 80g3 m2 m3 m4

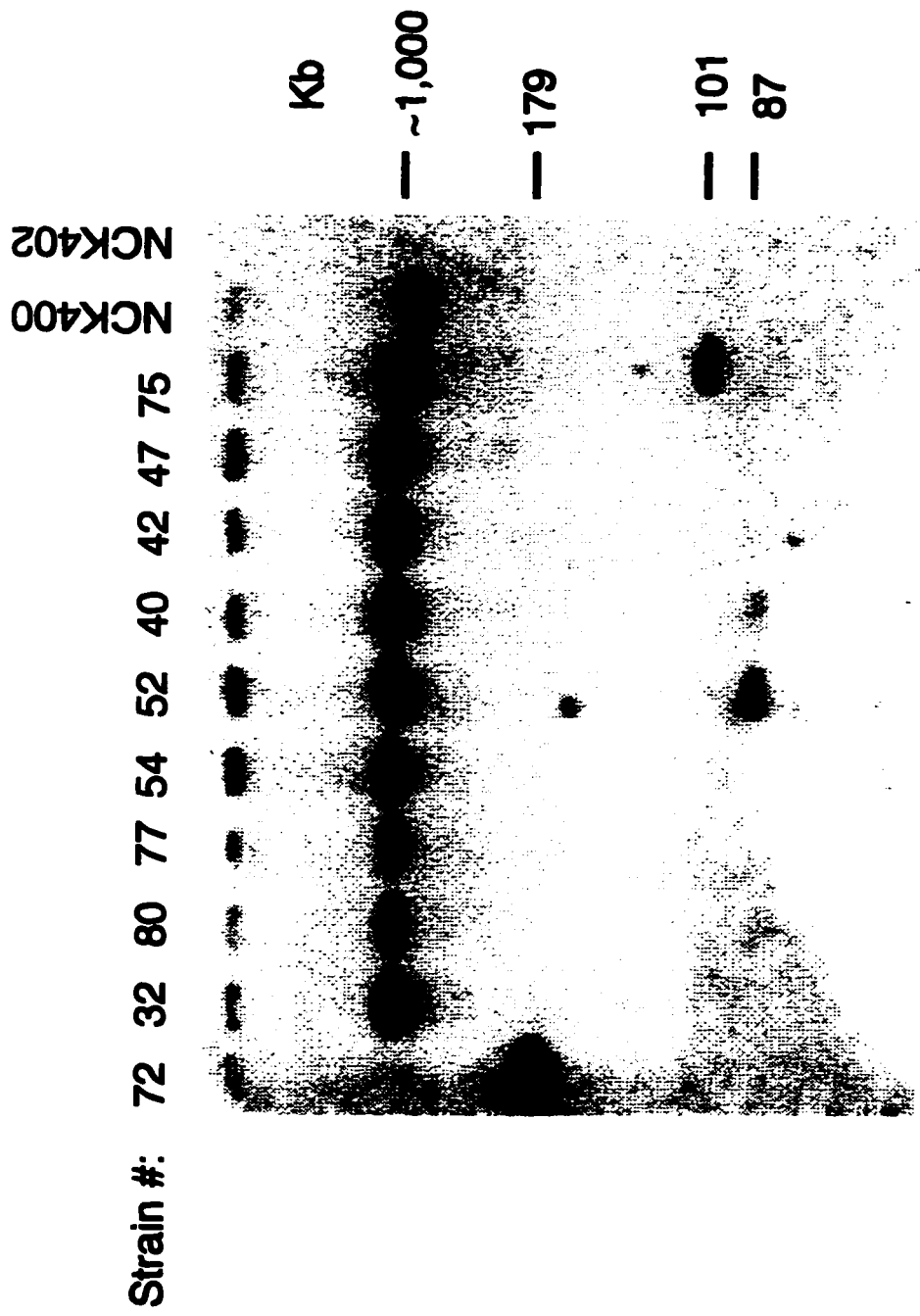


Kb

— 25

— 9

Fig. 13. Southern hybridization of *nisZ* probe (311 bp) to *Sma*I digested genomic DNA of various bean-sprout isolates. NCK402 and NCK400 were nisin negative and positive strains of *L. lactis* subsp. *lactis*, respectively.



3.6 Nisin gene copy number

Southern blotting with the *Eco*RI digested genomic DNA of isolate 80 demonstrated the presence of two bands (9 and 7.5 Kb) after hybridization with the 311 bp nisin Z structural gene probe which consisted of the whole *nisZ* gene, plus two flanking regions, 87 bp upstream and 50 bp downstream (Fig. 1). To verify if there were two copies of the nisin structural gene, a 120 bp probe containing only parts of the *nisZ* gene was used to hybridize against *Eco*RI digested chromosomal DNA of isolate 80. Only one band (9 kb) was evident after hybridization, indicating that only one copy of the nisin structural gene exists in the chromosome of isolate 80 (Fig 14).

3.7 Anti-listerial activity of bean-sprout isolate 80

3.7.1 Anti-listerial spectrum

The anti-listerial activity of isolate 80 against different species of *Listeria* and different strains of *L. monocytogenes* was tested (Table 9). When assayed with the deferred antagonism method, isolate 80 showed strong inhibitory effects against *L. monocytogenes*, *L. ivanovii*, *L. grayi*, *L. murrayi*, *L. seeligeri*, *L. welshimeri* and *L. innocua*. *L. monocytogenes* strain Scott A and *L. grayi* and *L. murrayi* were the most sensitive strains. When tested by the well-diffusion method, *L. monocytogenes* strain Scott A and *L. murrayi* were more sensitive than other species or strains tested.

3.7.2 Comparison of the anti-listerial activity of bean-sprout isolate 80 and bacteriocin producing *E. faecium*

Since some strains of *L. monocytogenes* were not as sensitive as others to the bacteriocin secreted by bean-sprout isolate 80, experiments were performed to compare the

anti-listerial activity of the bean sprout isolate with other lactic acid bacteria. This was done in order to search for a different combination of bacteriocin producing starter cultures for future use. A bacteriocin producing strain of *E. faecium* (ATCC 19434) was found to have weak inhibitory effects against strain Scott A, but strong activity against some other strains of *L. monocytogenes*. In contrast, bean-sprout isolate 80 had strong inhibitory activity against strain Scott A (Table 10).

3.7.3 Inhibition of *L. monocytogenes* by bean sprout isolate 80 *in vitro*

The initial inoculum of *L. monocytogenes* Scott A was 10^3 cells mL⁻¹ in all experiments. Isolate 80 was inoculated at both 10^5 (low inoculum) and 10^7 cells mL⁻¹ (high inoculum). At 10°C, with a low or high inoculum of isolate 80, *L. monocytogenes* was undetectable after 2 or 5 d of incubation. With the high inoculum of isolate 80 at 4°C, there was a slow and steady decline in levels of *L. monocytogenes*; after 24 d, the organism was undetectable. With the low inoculum at 4°C, *Listeria* levels were decreased slightly over the 24 d storage period, while that of the control increased to 5×10^4 cfu mL⁻¹ (Fig. 15).

3.7.4 Inhibition of *L. monocytogenes* by bean-sprout isolate 80 in Caesar salad

When co-inoculated with 10^2 cells g⁻¹ of *L. monocytogenes* on Caesar salad, as compared to the control, bean-sprout isolate 80 (10^8 cells g⁻¹) reduced the numbers of *L. monocytogenes* by 1 to 1.4 log cycles after storage for 10 d at 10 and 7°C, respectively (Fig 16). The combination of two bacteriocin producing starter cultures, i.e., *E. faecium* and bean-sprout isolate 80, significantly reduced the number of *L. monocytogenes* by 1.5 and 1.0 logs after storage for 10 d at 10 and 7°C, respectively, as compared to the control (Fig. 16).

3.7.5 Growth of lactic acid bacteria in Caesar salad

After storage at 7 or 10°C for 7 to 10 d, the numbers of bean sprout isolate 80 and/or *E. faecium* in Caesar salad remained stable when inoculated at an initial level of 10^8 cells g^{-1} (data not shown).

3.7.6 Headspace atmosphere in Caesar salad

Headspace CO₂ levels of both the control and treatment groups were similar, being 5-8% at day 0 and increasing to about 12-18% by the end of each experiments (10 d). Initial levels of O₂ were 10-15% and decreased to 1-5% by day 10 of the experiments (data not shown).

3.7.7 Change of pH in Caesar salad

The pH of the samples in the control and treatment groups were similar, being stable at pH 6.3 ± 0.002 (data not shown).

Fig. 14. Southern hybridization of *nisZ* probe to *EcoRI* digested genomic DNA of bean-sprout isolate 80. Lane 1: hybridization with *nisZ* structural gene internal probe (120 bp). Lane 2: hybridization with 311 *nisZ* structural gene probe which consisted of the whole *nisZ* gene, plus two flanking regions, 87 bp upstream and 50 bp downstream. Lane 3: *EcoRI* digested genomic DNA of bean-sprout isolate 80 before Southern hybridization. Lane m: DNA 1-kb ladder marker (GIBCO BRL).

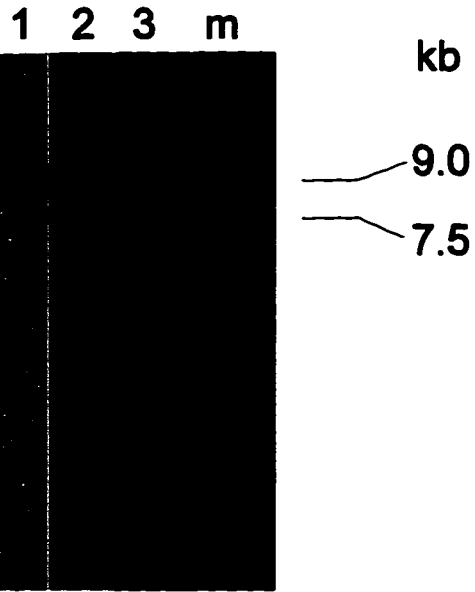


Table 9. Anti-listerial spectrum of bean sprout isolate 80

Indicator Strains	Inhibition zone (mm) ^a	
	Well-diffusion method	Deferred-antagonism method
<i>L. monocytogenes</i> Scott A, serotype 4b (HPB 3)	21.0±0.1	30.0±0.3
<i>L. monocytogenes</i> , cabbage isolate (HPB 845)	11.0±0.1	24.0±0.2
<i>L. monocytogenes</i> , beet isolate (HPB 534)	11.0±0.1	27.0±0.2
<i>L. monocytogenes</i> , coleslaw isolate, serotype 4b(HPB 2)	11.5±0.2	27.0±0.3
<i>L. monocytogenes</i> , bean isolate (HPB 540)	11.0±0.0	23.0±0.2
<i>L. monocytogenes</i> , chicken isolate, serotype 1/2a (HPB 564)	11.0±0.1	22.0±0.1
<i>L. monocytogenes</i> , ATCC15313 (305)	11.0±0.2	26.0±0.2
<i>L. ivanovii</i> (HPB 28)	12.5±0.1	25.0±0.1
<i>L. grayi</i> (HPB 29)	14.5±0.2	31.0±0.1
<i>L. murrayi</i> (HPB 30)	19.0±0.3	30.0±0.2
<i>L. seeligeri</i> (HPB 62)	11.0±0.1	25.0±0.3
<i>L. welshimeri</i> (HPB 92)	10.0±0.2	23.0±0.1
<i>L. innocua</i> (HPB 124)	11.0±0.1	25.5±0.1

^a Values are the means ±SE of four samples from two experiments.

Table 10. Comparison of the anti-listerial activity of the supernatant of bean-sprout isolate 80 and *E. faecium* ATCC19434

Indicator strains	Inhibition zone (mm) ^a			
	Well-diffusion method		Deferred-antagonism method	
	Bean-sprout isolate 80	<i>E. faecium</i>	Bean-sprout isolate 80	<i>E. faecium</i>
<i>L. monocytogenes</i> Scott A, serotype 4b (HPB 3)	21.0±0.2	19.5±0.1	36.5±0.4	26±0.3
<i>L. monocytogenes</i> , cabbage isolate (HPB 845)	11.0±0.1	20.0±0.1	22.0±0.2	22±0.2
<i>L. monocytogenes</i> , beet isolate (HPB 534)	11.0±0.0	26.0±0.2	20.0±0.2	24±0.3
<i>L. monocytogenes</i> , coleslaw isolate, serotype 4b(HPB 2)	11.5±0.1	21.5±0.1	25.0±0.2	23±0.1
<i>L. monocytogenes</i> , bean isolate (HPB 540)	11.0±0.2	20.5±0.3	21.0±0.1	23±0.1

^aValues are the means of duplicate samples from two experiments.

Fig. 15. Inhibition of *L. monocytogenes* Scott A by bean sprout isolate 80 in buffered MRS broth at 4 and 10°C. Solid bar: *L. monocytogenes* (10^3 cells mL⁻¹) control. Open bar: Both *L. monocytogenes* (10^3 cells mL⁻¹) and bean-sprout isolate 80 (10^5 cells mL⁻¹) were inoculated. Hatched bar: Both *L. monocytogenes* (10^3 cells mL⁻¹) and bean-sprout isolate 80 (10^7 cells mL⁻¹) were inoculated. With high inoculum of bean-sprout isolate 80, *L. monocytogenes* were undetectable after 2 d incubation at 10°C and 24 d incubation at 4°C. With low inoculum of bean-sprout isolate 80, *L. monocytogenes* were undetectable after 5 d incubation at 10°C. Values represent the means of two samples from two experiments.

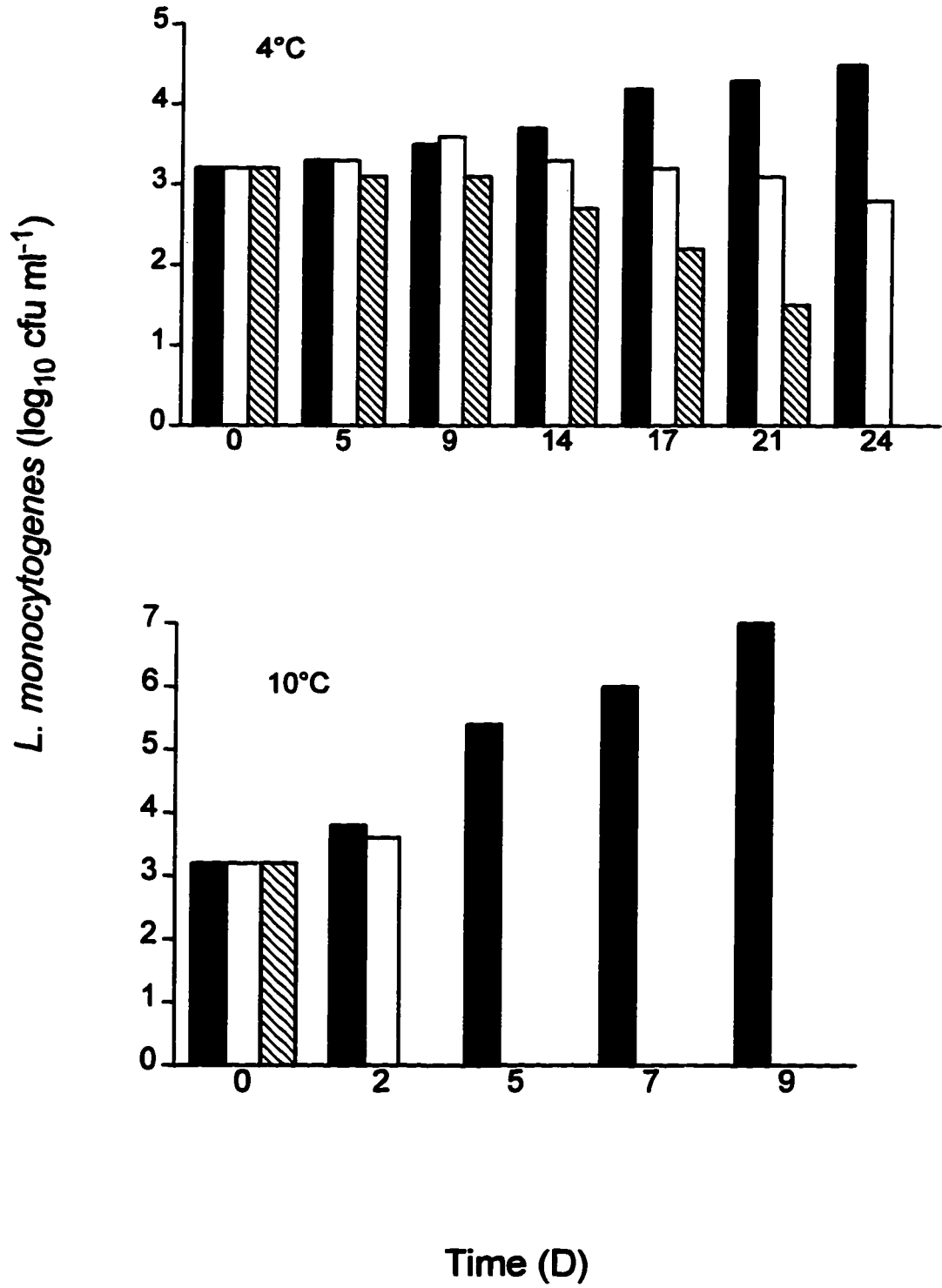
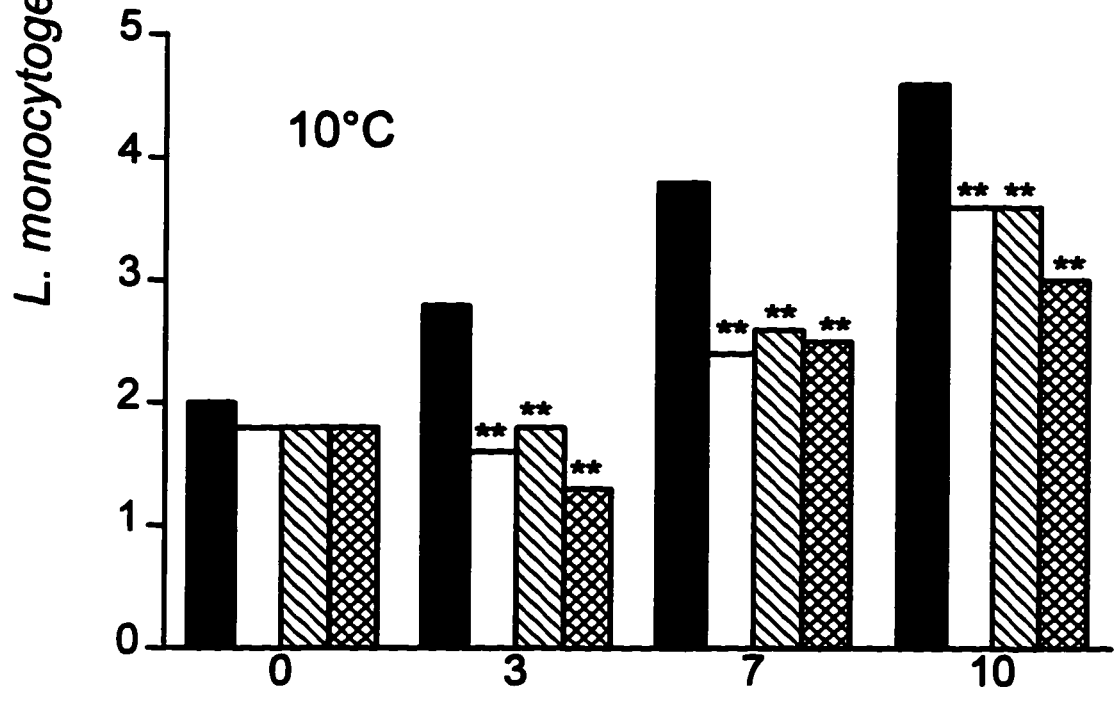
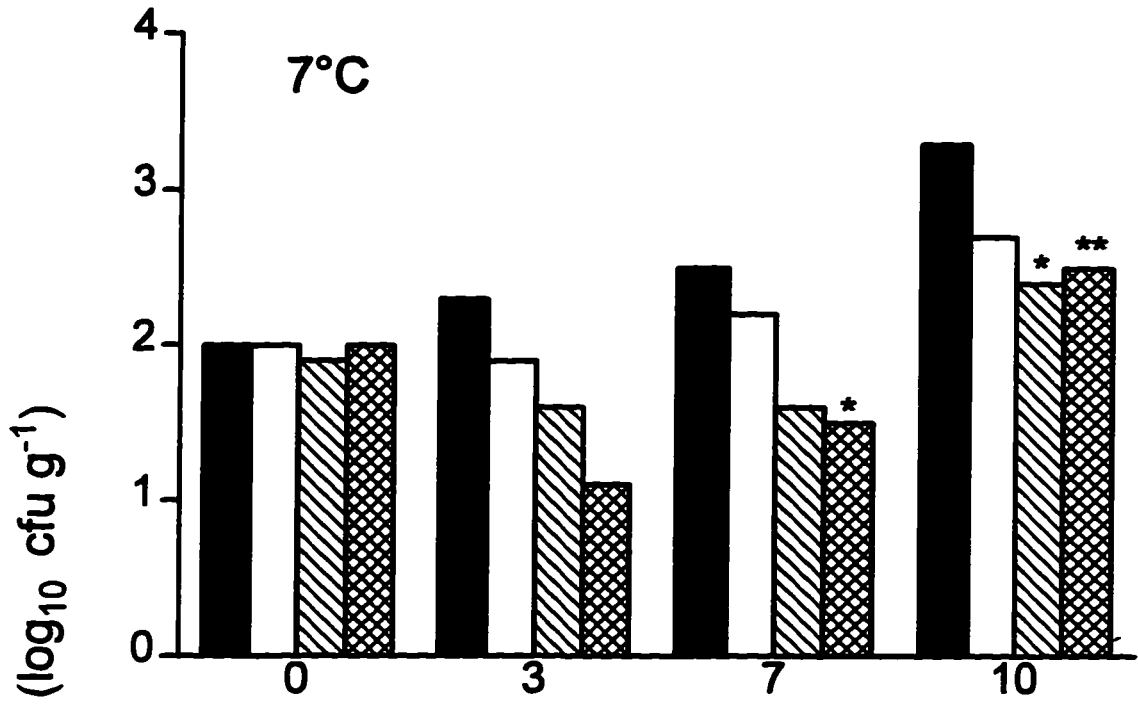


Fig. 16. Inhibition of *L. monocytogenes* by bean sprout isolate 80 in fresh-cut-ready-to-eat Caesar salad at 7 and 10°C. Solid bar: *L. monocytogenes* (10^2 cells g^{-1}) control. Open bar: *L. monocytogenes* (10^2 cells g^{-1}) and bean-sprout isolate 80 (10^8 cells g^{-1}) were inoculated. Hatched bar: *L. monocytogenes* (10^2 cells g^{-1}) and *E. faecium* (10^8 cells g^{-1}) were inoculated. Cross bar: *L. monocytogenes* (10^2 cells g^{-1}) and bean-sprout isolate 80 (10^8 cells g^{-1}) plus *E. faecium* (10^8 cells g^{-1}) were inoculated. Values represent the means of four to five samples from duplicate experiments. * $P < 0.05$, ** $P < 0.01$ (compared to control group).



Time (D)

4. DISCUSSION AND CONCLUSION

Based on the results of 16S rRNA sequencing and phenotypic tests, bean sprout isolate 80 was confirmed to be *L. lactis* subsp. *lactis*. Nine other bean sprout isolates (strains 32, 40, 42, 47, 52, 54, 72, 74 and 77) were also considered as *L. lactis* subsp. *lactis*. All ten strains produced nisin as determined by PCR amplification of the nisin structural gene and Southern hybridization with the nisin Z structural gene probe. They had identical antibiograms and the major biochemical characteristics of *L. lactis* subsp. *lactis* (Holt et al., 1994b). However, the ten isolates were found to be 5 different subtypes based mainly on plasmid profiles and PFGE banding patterns.

In this study, it was difficult to identify the bean-sprout isolates by biochemical tests. The initial identification of the bean sprout isolates by the VITEK system was incorrect, and led to some early erroneous conclusions. Thus, one should be very careful when using systems such as this for identifying organisms for which the database is limited or absent.

The bacterial species is the basic unit of bacterial taxonomy (Wayne *et al.*, 1987), and is defined as a group of strains, including the type strain, sharing 70% or greater DNA-DNA relatedness with a ΔT_m of 5°C or less. However, Vandamme *et al.* (1996) indicated that practical problems exist because different methods are used to determine the level of DNA-DNA homology. These methods do not always give the same (quantitative) results, and the value of 70% DNA relatedness seems only to be indicative rather than absolute. Vandamme *et al.* (1996) suggested that the bacterial species appears to be an assemblage of isolates which are characterized by a certain degree of phenotypic consistency, a significant degree of DNA-DNA hybridization and over 97% 16S rRNA sequence homology.

The 16S rRNA similarity analysis has become an important tool in bacterial taxonomy (Vandamme *et al.*, 1996). It is now generally accepted that rRNA is the best target for studying phylogenetic relationships because it is present in all bacteria, is functionally constant, and is composed of highly conserved as well as more variable domains (Schleifer and Ludwig, 1989; Stackebrandt and Goebel, 1994; Woese, 1987). The conserved sequences provide binding sites for primer elongation sequence protocols (Giovannoni, 1988; Olsen, 1986). Other regions of the 16S rRNA are unique to particular organisms or groups of related organisms. This situation offers the opportunity to design specific hybridization probes to identify an organism or a group of organisms (DeLong *et al.*, 1989; Giovannoni *et al.*, 1988; Olsen *et al.*, 1986). Direct sequencing of parts or nearly the entire 16S or 23S rRNA molecules by PCR provides a phylogenetic framework which serves as the backbone for modern microbial taxonomy (Vandamme *et al.*, 1996). It is now possible to identify *L. lactis* subsp. *lactis* by 16S rRNA sequencing because the 16S rRNA sequences of this organism, as well as many other organisms have been published (Collins *et al.*, 1989; Salama *et al.*, 1991; Chiaruttini and Milet, 1993).

Some molecular biology methods have been developed to identify *L. lactis* subsp. *lactis* in natural ecosystems. Klijn *et al.* (1991, 1995) identified *Lactococcus* species from natural ecosystems using identification methods based on PCR amplification and species-specific probes derived from 16S rRNA sequences. Salama *et al.* (1991) developed two classes of phylogenetic probes, a subspecies-specific rRNA probe for *L. lactis* subsp. *cremoris* and a genus-specific rRNA probe for the lactococci. This study is the first to identify *L. lactis* subsp. *lactis* from food samples by sequencing 16S rRNA gene using a

PCR technique, which appears to be direct and a less time consuming method.

Ribotyping involves the use of Southern blot analysis to detect polymorphisms in chromosomal regions containing ribosomal RNA genes. Classic ribotyping uses labelled probes containing *E. coli* 16S + 23S rRNA sequences (Stull *et al.*, 1988). Helio *et al.* (1995) concluded that ribotyping has three major advantages: a) the genes coding for rRNA are highly conserved, allowing the use of a single probe to subtype all eubacteria; b) since most bacteria contain multiple rRNA genes, a reasonable number of bands are obtained after probing; and c) all strains have at least one rRNA operon. However, ribotypes are a relatively stable characteristic within a species, and epidemiologically unrelated isolates may demonstrate identical patterns (Gordillo *et al.*, 1993; Maslow *et al.*, 1993; Prevost *et al.*, 1992). In contrast to the classic view that ribotyping was only a subtyping method, Webster (1995) indicated that the RiboPrint™ system, an automated ribotyping method, could provide accurate speciation and type descriptions of foodborne microorganisms without a presumptive identification step. In this study, by using the RiboPrint™ system, it was possible to identify the lactococci at the subspecies level without a presumptive identification step. Presumably, because ribotyping is considered mainly as a typing method, and because of its intensive labour, there have been no reports on using ribotyping to identify *L. lactis* from food samples. However, the RiboPrint™ system could be a useful tool for identifying LAB.

In order to assess the discriminatory ability of typing systems, Hunter and Gaston (1988) suggested the use of a single numerical index of discrimination (D), based on the probability that two unrelated strains sampled from the test population will be placed into

different typing groups. This probability can be calculated by Simpson's index of diversity, which was developed for the description of species diversity within an ecological habitat (Simpson, 1949). The D was calculated for each of the typing methods (Table 5). The Simpson's Index of Discrimination was low for PFGE and plasmid typing in this study. This may be due to the fact that most of the strain tested belonged to the same subtype.

PFGE has previously been shown to be a simple and reliable method for typing of lactococcal strains (Le Bourgeois *et al.*, 1989; Tanskanen *et al.*, 1990). In this study, the discriminatory ability of PFGE with *SmaI* was lower than that of plasmid typing (Table 5). Aside from discriminatory ability, in order to fully assess which typing method is the most efficient, reproducibility must also be considered. Future work in this area should include a larger number of different LAB strains, and the use of more restriction enzymes for PFGE.

The presence of different plasmid populations in different lactococcal strains has been documented (Anderson and McKay, 1983; Davies *et al.*, 1981; Frère, 1994; O'sullivan and Klaenhammer, 1993). The present study demonstrated some of the different plasmids that can be found in these organisms. Plasmid profile analysis has been considered as a poor typing method, since plasmids are mobile extrachromosomal elements and are not part of the chromosomal genotype that defines the host strain (Abeit, 1995).

The present study demonstrated that it was not possible to differentiate between the bean-sprout isolates by the use of antibiograms, implying that the antibiotic resistance genes in bean-sprout isolates are relatively stable. The plasmid-less derivative of bean-sprout isolate 32 contained the same antibiogram as the wild type isolates, indicating that the antibiotic resistance genes were most likely located on the chromosome.

As for biotyping of lactic acid bacteria, there are no published schemes available. In the latest edition of Bergey's Manual of Determinative Bacteriology, Holt *et al.* (1994b) indicated that 90% or more strains of *L. lactis* subsp. *lactis* fermented lactose but not raffinose, and that 80% of the strains are mannitol-negative. The present study found that 80% (2/10) of the bean-sprout isolates were lactose-positive and raffinose-negative, while half (5/5) of the isolates were mannitol-negative. The fermentation of these sugar could be considered in the future as part of a biotyping scheme for *L. lactis* subsp. *lactis*.

The natural habitat of *L. lactis* strains has been the subject of much debate. Although these organisms are commonly found in milk and dairy products, Stark and Sherman (1935) believed that their natural habitat was plant material. Mundt (1970) pointed out the possibility of a relatively recent adaptation to milk. Since nisin was first characterized, it has been generally believed that this bacteriocin occurs naturally only in dairy products. This traditional view has been challenged by the recent isolation and characterization of two nisin-producing *L. lactis* subsp. *lactis* strains from a commercial sauerkraut fermentation (Harris *et al.*, 1992). More recently, Rodrigues *et al.* (1995) also reported the isolation of nisin-producing *L. lactis* strains from dry fermented sausages. The present study demonstrated that fresh bean sprouts can also be a source of nisin producing *L. lactis* subsp. *lactis* strains. Among the 76 bacterial isolates tested for bacteriocin activity in this study, 23 were found to produce anti-listerial compounds, 10 of which were confirmed to produce nisin. All the isolates were different from the *L. lactis* subsp. *lactis* strain NCK400, isolated from sauerkraut (Harris, 1992).

Based on DNA sequence analysis, the chromosomally located nisin structural genes

of bean-sprout isolates 80 and 32 were identified as *nisZ*. The difference in protein sequence between nisin Z and nisin A lies only at amino acid position 27, where asparagine is present in nisin Z and histidine in nisin A (Mulders *et al.*, 1991). DNA sequence analysis of the respective nisin genes revealed that a simple base substitution was responsible for the change in nisin structure (Mulders *et al.* 1991). It has been reported that some *L. lactis* strains isolated from dairy products and fermented vegetables can produce nisin Z (Graeffe *et al.*, 1991; Mulders *et al.*, 1991; De Vos *et al.*, 1993).

Some lactic acid bacteria have been reported to produce bacteriocins inhibitory to *L. monocytogenes*. These include *C. piscicola* (Ahn and Stiles, 1990; Mathieu *et al.*, 1994; Milliere *et al.*, 1994; Schillinger and Holzpfel, 1990; Stoffels *et al.*, 1992; Stoffels, 1993), *E. faecalis* (Villani *et al.*, 1993), *E. faecium* (Arihara *et al.*, 1993; Farias *et al.*, 1994; Kato *et al.*, 1993; McKay, 1990; Olasupo *et al.*, 1994; Parente and Hill, 1992; Vlaemynck *et al.*, 1994), *L. acidophilus* (Kanatani *et al.*, 1995), *Lactobacillus bavaricus* (Winkowski *et al.*, 1993; Winkowski and Montville, 1992), *Lactobacillus curvatus* (Ming and Daeschel, 1993; Sudirman *et al.*, 1993; Tichaczek *et al.*, 1992), *Lactobacillus plantarum* (Atrih *et al.*, 1993; Fricourt *et al.*, 1994), *Lactobacillus sake* (Schillinger and Lücke, 1989; Sobrino *et al.*, 1991; Tichaczek *et al.*, 1992), *Leuconostoc carnosum* (Keppler *et al.*, 1994; van Laack *et al.*, 1992), *Leuconostoc gelidum* (Hastings and Stiles, 1991), *Leuconostoc mesenteroides* (Daba *et al.*, 1991; Hechard *et al.*, 1992; Mathieu *et al.*, 1993), *Pediococcus acidilactici* (Berry *et al.*, 1990), *Pediococcus pentosaceus* (Spelhaug and Harlander, 1989), and *Lactococcus lactis* (Spelhaug and Harlander, 1989). However, most of these strains were isolated from meat or dairy products and not much effort has been expended to isolate such organisms from

vegetable products. Only a few anti-listerial lactic acid bacteria isolated from fermented vegetables, e.g., *L. mesenteroides* subsp. *mesenteroides*, *Leuconostoc paramesenteroides*, *Pediococcus pentosaceus* (Kim, 1995) and *E. faecium* (Ha *et al.*, 1994) isolated from kimchi (a Korean-style fermented vegetable) and *L. lactis* subsp. *lactis* (Harris, 1992) from sauerkraut were found to inhibit *L. monocytogenes*. Mung bean sprouts were found to contain anti-listerial activity, probably due to LAB (Aytac and Gorris, 1994). In this study, we found that the anti-*L. monocytogenes* activity was from nisin-producing LAB. To our knowledge, this study showed for the first time that anti-listerial lactococci exist in fresh vegetables.

Compared with the bacteriocin producing *E. faecium* (ATCC 19434), bean-sprout isolate 80 had a stronger inhibitory effect against *L. monocytogenes* strain Scott A as tested by both the well-diffusion and deferred antagonism methods. Both LAB strains had similar activity against other *L. monocytogenes* pathogenic strains when tested with the deferred antagonism methods. However, with the well-diffusion method, *E. faecium* was more inhibitory than bean-sprout isolate 80 against *L. monocytogenes* strains other than Scott A. There has been only one report on the differences in sensitivity of various *Listeria* strains to bacteriocins. Kim (1995) recently showed that *L. ivanovii* 28, *L. monocytogenes* Scott A3 and *L. monocytogenes* ATCC 19116 were more sensitive than other *Listeria* strains to *Pediococcus* and *Leuconostoc* spp. isolated from kimchi.

Most of the reports on isolating anti-*L. monocytogenes* bacteriocin producing bacteria used strain Scott A as the indicator organism. Since the sensitivity to different bacteriocins is different among various strain of *L. monocytogenes*, it is important to use various strains

of *L. monocytogenes* as indicators.

Although the underlying reasons for these differences in bacteriocin sensitivity are unknown, the different structure or function of the cell membrane among different species/strains may be involved. The biochemistry of the cell structure of *L. monocytogenes* and other *Listeria* spp. was studied by Fiedler (1988), who proposed a macromolecular model of the organization of the *Listeria* cell wall. Electron micrographs of the cell wall showed it to be that of a typical Gram-positive bacteria, i.e., a thick homogeneous structure surrounding the cytoplasmic membrane and without the outer membrane characteristics of Gram-negative bacteria. *Listeria* cell walls are composed of about 35% peptidoglycan, consisting of cross-linked *meso*-diaminopimelic acid. The remaining carbohydrates are part of the cell wall teichoic acids, which are polymers covalently linked to a specific site on the peptidoglycan. They are usually composed of glycerol or ribitol, neutral sugars, *N*-acetylamino sugars, and phosphate. Structurally, two types of cell wall teichoic acids exist amongst *Listeria* serotypes. The first type consists of ribitol residues covalently linked by phosphodiester bonds between C-1 and C-5 and are sometimes found with *N*-acetylglucosamine substituted at C-2. This type is found associated with serotypes 1/2a, 1/2b, and c, 3a, 3b, and 3c, and 7. In the second type, *N*-acetylglucosamine is integrated into the chain. This type is found associated with serotypes 4a, 4b, and 4d.

It has been noticed that there is a difference in the cell membrane function between the bacteriocin sensitive and resistant bacteria, which may help to explain the different sensitivities observed among the different species or strain of *Listeria*. Harris *et al.* (1991) and Ming and Daeschel (1993) observed nisin-resistant variants of *L. monocytogenes*

appearing at frequencies of 10^{-6} to 10^{-8} . Spontaneous resistance may develop from an alteration and/or mutation of the cell-surface or cell-membrane molecular constituents with which bacteriocins interact (Muriana,1996). Ming and Daeschel (1993) examined a spontaneous nisin-resistant isolate of *L. monocytogenes* Scott A that had exhibited a different growth rate and variations in membrane fatty acid composition, as compared to that of the wild-type strain. Ming and Daeschel (1995) further found that the nisin-resistant *L. monocytogenes* Scott A strain had significant reductions in three phospholipids (phosphatidylglycerol, diphosphatidylglycerol, and bis-phosphatidylglyceryl phosphate) as compared to the nisin-sensitive wild-type strain. In addition, the cell surface of resistant cells was less hydrophobic than sensitive cells.

Although further work needs to be done to investigate the mode of action of bacteriocins, the fact that a large variation in sensitivity to nisin exists among various strains of *L. monocytogenes* suggests that it may be more beneficial to use a combination of different bacteriocins, or a combination of different lactic starter cultures.

Harris *et al.* (1992) reported that a 4 Kb probe, which consisted of the nisin structural gene plus flanking sequences, hybridized to two *EcoRI* generated chromosomal DNA fragments of a sauerkraut isolate. However, they did not determine whether the second fragment was another copy of the gene or simply a region of homology within the 4 Kb region. Since *EcoRI* sites exist only external to *nisZ* on the nisin transposon (Rauch and De Vos, 1992), it was postulated that bean-sprout isolate 80 might have two copies of *nisZ* when two fragments of *EcoRI* digested chromosomal DNA hybridized with the *nisZ* 311 bp probe. However, after an internal probe (120 bp) was used in the hybridization, it was confirmed

that isolate 80 had only one copy of the *nisZ* gene. It is recommended that only an internal region of the nisin structural gene be used as a probe to confirm the nisin gene copy number.

For the following reasons, the LAB from this study are potentially useful as anti-*L. monocytogenes* agents for use on FCV:

a) Nisin is regarded as non-toxic when consumed orally and has proven to be a safe food preservative (Delves-Broughton,1990). The susceptibility of nisin to enzymatic degradation is an advantage for its use in food, as nisin is quickly digested and would not effect the intestinal flora or be absorbed into the bloodstream (Molitor and Sahl, 1991). In contrast, the susceptibility of nisin to enzymatic inactivation is also a disadvantage, as nisin levels in food can decrease quickly due to degradation by food enzymes. This disadvantage could be avoided by using starter cultures which express nisin continually. At the current time, nisin has been approved for use in approximately 50 countries, including the USA, the United Kingdom and China (Delves-Broughton and Gasson, 1994). In 1988, it was given GRAS status (generally recognized as safe) by the U.S. FDA (FDA, 1988).

b) The isolates are part of the indigenous flora of a fresh vegetable. Aytac and Gorris (1994) reported that the initial levels of LAB in fresh mung-bean sprout were 10^6 cfu g⁻¹ and reached 10^9 cfu g⁻¹ after storage at 4 or 7°C for 7 d. In the present study, after inoculating different levels of bean-sprout isolates onto Caesar salad, the maximum numbers of the organism recovered were 10^8 cfu g⁻¹ after storage at 7 or 10°C for 7 to 10 d, lower than that in the fresh mung-bean sprouts.

c) Mastsusaki *et al.* (1996) reported that the production of nisin Z was optimal at 30°C and in the pH range 5.0-5.5. The inoculation of LAB to produce nisin "*in situ*" would

require a nisin-producing strain that can grow and produce the bacteriocin at refrigeration temperatures (Lücke and Earnshaw, 1991; Hastings and Stiles, 1991). The bean-sprout isolates can grow, produce nisin and inhibit *L. monocytogenes* at low temperatures (4 - 10°C), which would be suitable for FCV preservation in refrigeration temperatures.

Muriana (1996) concluded that most food applications of bacteriocins to-date have produced only modest reductions of 1 to 3 logs in numbers of *L. monocytogenes*. Although these levels are not acceptable as a primary preservation method, they are useful as a primary hurdle for controlling foodborne pathogens. The use of nisin-producing starter cultures has also recently been reported in food preservation using a very high inoculum of nisin-producing *L. lactis* subsp. *lactis*. Wong *et al.* (1986) used *L. lactis* subsp. *lactis* (10^9 cells g⁻¹) to extend the shelf-life of vacuum-packed frankfurter sausages held at 7°C for 6 weeks. They found that the growth of aerobic and psychrotrophic bacteria was 1 to 2 log cycles lower than the uninoculated control. The present study was the first attempt at using starter cultures for FCV preservation. The results indicated that the bean-sprout isolates could be used as an extra hurdle to inhibit the growth of *L. monocytogenes* in fresh-cut-ready-to-eat Caesar salad.

The reasons that the anti-listerial effects of the bean-sprout isolate in Caesar salad were not as effective as that in MRS broth needs further investigation. Mastsusaki *et al.* (1996) reported that the production of nisin Z was optimal at 30°C and in the pH range 5.0-5.5. The bean-sprout isolates in this study grew and expressed bacteriocin in both of relatively low temperatures, however, the pH (6.3 ± 0.002) of the salad samples was probably not optimal for nisin production. In addition, the distribution of the bacteriocins in

vegetables may not be as uniform as in liquid medium and therefore the contact between bacteriocin and *L. monocytogenes* is much better in broth than in Caesar salad.

Future work could be done to improve the anti-listerial effects of LAB in FCV by adding other barriers or hurdles. Leistner (1995) indicated that the microbial stability and safety of most foods should be based on a combination of several factors (hurdles), which combine either additively or synergistically to control microbial growth in foods. This is illustrated by the so-called hurdle effect. Wimpfheimer *et al.* (1990) reported that under a modified atmosphere of 75% CO₂, 25% N₂, both *L. monocytogenes* as well as other aerobic bacteria failed to grow on raw chicken stored at either 4, 10 or 27°C. Although, in the present study, CO₂ levels in the headspace atmosphere of the salad samples gradually increased from 5-8% to 12-18% after 10 d storage at 7 or 10°C; this level would not be sufficient to inhibit growth of *L. monocytogenes*. Using predictive modelling, Farber *et al.* (1996) found that *L. monocytogenes* grew in the presence of 50% CO₂ at 7°C. However, higher headspace CO₂ levels combined with the use of LAB would create more effective hurdles to the growth of *L. monocytogenes* on FCV. Other hurdles that could be considered to be used together with LAB to inhibit the growth of *L. monocytogenes* include irradiation (Loaharanu, 1995), pulsed electric field technology (Grahl and Märkl, 1996; Pothakamury *et al.*, 1995) and far-infrared irradiation (Hashimoto *et al.*, 1992).

In summary, in this study I isolated and fully characterized bacteriocin producing *L. lactis* subsp. *lactis* strains from bean sprouts. The bacteriocin produced was found to be nisin Z. One of the isolates (number 80) was tested for anti-listerial activity *in vitro* (MRS broth) and *in vivo* (Caesar salad) and found potentially useful as a hurdle to the growth of *L.*

monocytogenes in FCV Caesar salads.

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