Screening lactic acid bacteria for bacteriocin production against *Listeria monocytogenes* was carried out with 324 strains isolated from different sources. Four of the five producer strains were identified as *Lactococcus lactis* subsp. *lactis* and the fifth was a *Lactobacillus* species. Antimicrobial substance produced by three of the *Lc. lactis* strains was nisin. The fourth (lactococcin LBIIA) was unique and differed from nisin by sensitivity to trypsin and α-chymotrypsin, lack of inhibitory activity against *Micrococcus flavus*, and resistance to 16 hours of exposure at pH 10.0. The antimicrobial substance produced by the *Lactobacillus* sp. was not protein in nature; it also was not an organic acid, hydrogen peroxide, or a bacteriophage.

Nisin was lethal to nine strains of three species of *Listeria*, including pathogenic *L. monocytogenes*. Effect of nisin on *L. monocytogenes* in cottage cheese and yogurt was investigated. Nisin inhibited this
pathogen in both products. Fermentation of milk by *Streptococcus thermophilus* and *Lactobacillus bulgaricus* to produce yogurt was inhibited by nisin at concentrations higher than 50 IU/ml; less than 50 IU/ml could be used to inhibit growth of *L. monocytogenes* in yogurt and cottage cheese.

Use of nisin at 40 IU/ml to extend the shelf-life of Feta cheese manufactured with starter containing *S. thermophilus* and *L. bulgaricus* also was studied. In comparison to control cheese made without nisin, the nisin-containing cheese actually spoiled more rapidly, apparently by limiting the growth of *L. bulgaricus* and allowing contaminating Gram-negative psychrotrophs to grow with less competition.

Attempts to study bacteriocin production by dairy *Leuconostoc* (*Leu*) strains isolated from natural vegetation was hindered by the unavailability of a suitable selective isolation medium. Such an effective medium, was formulated containing 1% glucose, 1% bacto peptone, 0.5% yeast extract, 0.5% beef extract, 0.25% gelatin, 0.5% calcium lactate, 0.05% sorbic acid, 75 µg/ml sodium azide, 0.25% sodium acetate, 0.1% Tween 80, 15% tomato juice, 30 µg/ml vancomycin, 0.20 µg/ml tetracyline, 0.05% cysteine hydrochloride and 1.5% agar. Over 100 strains of *Leu.* cultures were easily isolated from dairy products and vegetables using this medium. Cultures of *Leu. dextranicum* and *Leu. cremoris* produced the same number of colonies in this medium as in MRS agar.
Application of Bacteriocins Produced by Lactic Acid Bacteria in preserving Dairy Products and Development of a Selective Medium for Leuconostoc Isolation

by

Noreddine Benkerroum

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APPROVED:

Redacted for Privacy

Professor of Microbiology in charge of major

Redacted for Privacy

Head of Department of Microbiology

Redacted for Privacy

Dean of Graduate School

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Typed by, Deborah Norbraten
DEDICATION

To my parents and my family
for their love.

To Mrs. Susan Sandine for her care.
To the Nelsons and the Bonys (France) for their friendship
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APPLICATION OF BACTERIOCINS PRODUCED BY LACTIC ACID BACTERIA IN PRESERVING DAIRY PRODUCTS AND DEVELOPMENT OF A SELECTIVE MEDIUM FOR LEUCONOSTOC ISOLATION

INTRODUCTION

Lactic acid bacteria (LAB) are a heterogeneous group of microorganisms consisting of rod, coccus and coco-bacillary shaped bacteria. They produce, from fermentable carbohydrates, lactic acid, either almost exclusively (homofermentative) or with other metabolic endproducts (heterofermentatives). They are also typically Gram-positive and catalase-negative, though some strains produce pseudocatalase. Their natural habitats are milk and dairy products, herbage, green vegetables, the intestinal tract and vagina (36, 45). Taxonomy of LAB has undergone considerable revision in recent years as microbial classification systems are being based on more powerful and reliable techniques such as computer assisted numerical taxonomy and molecular biology. Traditionally this group of microorganisms was composed of four genera: Streptococcus, Leuconostoc, Pediococcus and Lactobacillus. All belonged to the same family Lactobacillaceae. In the latest completely revised Bergey's Manual of Systematic Bacteriology (4), these bacteria were placed into two different families: Lactobacillus remained in the family Lactobacillaceae while the three other genera were placed in the family Streptococcaceae. The most important and recent change however, has been the establishment of a new genus, Lactococcus, to replace and expand the Lancefield serological group N Streptococcus genus. This genus has been known and used by dairy technologists
and scientists for almost a century, since the isolation of a pure culture of *Streptococcus lactis* in 1909 by Löhnis, as quoted by Teuber and Geis (45). The new *Lactococcus* taxon was suggested in 1985 by Schleifer et al. (37) and was validated in 1986 by the International Union of Microbiological Societies (1). Reasons for establishment of this new genus as well as detailed information about its characteristics and those of individual species are provided by Schleifer et al. (37) and Sandine (34). In addition to the four genera mentioned above, some microbiologists are inclined to consider *Bifidobacterium* as a fifth genus of LAB since its members also produce lactic acid from carbohydrates. They also are Gram-positive, catalase-negative and have the same natural habitat as most lactobacilli (5, 24). In fact, *Bifidobacterium* was for many years considered as a species of the genus *Lactobacillus* (5). Weiss and Rettger (47) even claimed it to be a biovariety of *Lb. acidophilus*, which has been strongly objected to by a number of contemporary investigators (5, 6, 29, 39). Therefore, further strain characterization was needed to provide data to serve as a basis for clarifying this matter. Briggs (6) and Sharp (39) studied 390 and 442 strains, respectively, for this purpose and they both found extreme variability among bifidobacterial strains as to their physiological and serological characteristics. It then became clear that they were dealing with a separate group of microorganisms, distinct from lactobacilli. Therefore, attempts were made to reclassify them into another family, but unsuccessfully since the physiological and serological tests used in these studies proved insufficient to provide definitive answers. Hence, bifidobacteria continued to belong to the
genus *Lactobacillus* as a single species called *Lb. bifidus* until the 1970's. As molecular biology techniques became available, bifidobacteria were placed as a separate genus *Bifidobacterium* in the family *Actinomycetaceae* on the basis of the moles percent of guanine and cytosine in their DNA and also on the basis of 16S ribosomal RNA homology. Other species of the genus *Streptococcus* (*Str.*) are also known as LAB, namely *Str. faecium* and *Str. faecalis* (9, 10, 31, 34) of the *Enterococcus* group and *Str. thermophilus* (34) and *Str. bovis* (23) of the so-called "Other Streptococci" group in the newly revised Bergey's Manual of Systematic Bacteriology (4). Previously those two species were placed in the Viridans streptococcal group (28, 34, 40), which no longer exists. *Str. thermophilus* has been extensively used in dairy fermentations in combination with other lactic acid producing bacteria, such as *Lc. lactis* subsp. *cremoris* (previously called *Str. cremoris*), in Cheddar cheese manufacture in Australia (9) and with other thermophilic LAB such as *Lb. bulgaricus*, *Lb. helveticus* or *Lb. acidophilus* in yogurt and Feta cheese manufacture (34, 30). *Str. faecalis* and *Str. faecium* also have been used in dairy fermentations (9, 31). These strains were shown to offer advantages over *Lactococcus* species in the manufacture of some cheeses, especially Cheddar cooked at high temperature (42°C). Some of these advantages were: higher resistance to cooking temperature and the salt level normally used, greater resistance to bacteriophages, and better genetic stability (9). Other investigations on the use of *Str. faecalis* and *Str. faecium* in Cheddar cheese showed that *Str. faecium* provided a better quality of cheese than the regular commercial starter, while *Str. faecalis*
contributed the same or a less desirable quality (31). However, the use of these two species in cheesemaking was highly controversial for two reasons: 1) they belong to the *Enterococcus* group, organisms from which are used as indicators of fecal contamination in foods (Jacquet, personal communication); 2) *Str. faecalis* is β-haemolytic and associated with urinary tract infections and subacute endocarditis (28). Also *Str. faecium* occasionally is associated with endocarditis and some strains produce biogenic amines resulting in scombroid disease (34). This contrasts with the fact that LAB are Generally Recognized as Safe (GRAS). However, according to Mundt (28) these two species are not in reality associated with fecal contamination. Moreover, β-haemolysin production by *Str. faecalis* and amine production by *Str. faecium* are plasmid mediated (28), so cured strains could be used in fermentations. This would allow non-haemolytic and non-amine producing variants of both species to be isolated and used in dairy fermentations. *Str. bovis* was also considered by Jones et al. (23) as a homofermentative lactic acid-producing bacterium. These authors showed that *Str. bovis* was a better inoculant in silage than commercial inoculants composed of homo and heterofermentative LAB. In fact, this species was reported to be the least fastidious in the genus *Streptococcus* (20). Therefore, it grew rapidly, resulting in a swift decrease of pH, with lactic acid as the major product from carbohydrate breakdown. These are ideal conditions in silage. Other LAB inoculants were shown to produce appreciable amounts of ethanol and acetic acid, which are not desirable in silage (23). To our knowledge, *Str. bovis* has not been used in dairy fermentations so far.
Although a detailed presentation on the recent taxonomic modifications in the genus *Streptococcus* will not be offered herein, it seems appropriate to mention some of these changes, especially those related to organisms used in the research described in this thesis. As mentioned earlier, the most important change recently made was the establishment of the new *Lactococcus* genus and its validation by the International Union of Microbiological Societies in 1986 (1). This genus was not recognized even in the latest completely revised Bergey's Manual of Systematic Bacteriology, (4) where the previous nomenclature of lactic streptococci was still used. It was mentioned, however, that the "transfer of all lactic streptococci (group N) to the genus *Lactococcus* is in reasonable agreement with findings from numerical taxonomy". The manual was probably edited before the new taxon was validated. Also, the lactic streptococci in the revised Bergey's Manual (4) consist of only two species: *Str lactis* and *Str. raffinolactis*, while in the new taxonomy proposed by Schleifer et al. (37) the *Lactococcus* (*Lc.*) genus, replacing the former *Streptococcus* (group N) genus, consists of 4 species: *Lc. lactis*, *Lc. garviæ*, *Lc. plantarum* and *Lc. raffinolactis*. Further changes also were made in the taxonomy of *Streptococcus* genus in this new Bergey's Manual. Because of the diversity of members of this genus and for convenience, Sherman (40) divided this genus into 4 groups: Pyogenic, Viridans, Lactic and Enterococcus. This division proved useful for over 50 years. Now, in the new Bergey's Manual, this genus is divided into 6 categories: Pyogenic, Oral, Enterococcus, Lactic, Anaerobic and Other Streptococci. New species, strictly anaerobic, were included in the Anaerobic group,
whereas, species perviously in the Viridans group were moved to the Other Streptococci group.

From the above discussion two main conclusions may be made:

1). The definition of LAB provided by Orla-Jensen in 1919 (29) and quoted by Kandler (24) is still used by workers today: "Gram-positive, non-sporing, microaerophilic bacteria whose main fermentation product from carbohydrate is lactate". This definition is rather vague. All streptococci, including pathogenic strains and even members of other genera, fall into this group as so defined. Also, Bifidobacterium species would be excluded since they are strict anaerobes. Therefore we propose the following modification which hopefully will provide well-defined boundaries for this group: LAB are non-pathogenic, Gram-positive, catalase-negative, non-spore-forming bacteria whose main product of carbohydrate fermentation is lactic acid.

2) In view of the recent taxonomic changes, LAB are now composed of the following genera:

- *Lactococcus* (*Lc.*), all species included.
- *Lactobacillus* (*Lb.*), all species included.
- *Pediococcus* (*Pd.*), all species included.
- *Leuconostoc* (*Leu.*), all species included.
- *Bifidobacterium* (*B.*), all species included.
- *Streptococcus* (*Str.*), only characterized, non-pathogenic strains of Enterococcus and Other Streptococci.
This preoccupation with the taxonomy of LAB is not without justification. Indeed LAB have been most useful to humans from the beginning of mammalian life. A long time before their existence was even suspected, ancient civilizations were using them in their food supplies either for preservation or to enhance the quality of some foods (38). Cheesemaking practices may be the best and the oldest example of the use of LAB in foods during early civilization dating from 6000 to 7000 BC (38). Now that LAB are well known and well characterized, they are being found to be of genuine benefit to the well-being of humans.

LAB are used in the fermentation of many foods, including milk, green olives, cucumbers, cabbage, etc. (8, 12, 16, 45), meats, (2, 41, 42), wine (22, 25) and other products (42, 44). Their primary role in the fermentation process is the production of lactic acid with a concomitant pH decrease. However, they may play other roles, namely inhibition of undesirable bacteria (e.g. spoilage or pathogenic bacteria), and enhancement of the texture and the flavor. In addition to lactic acid, they also produce a variety of aromatic substances including acetic acid, ethanol, diacetyl, acetaldehyde, and dimethyl sulfide (44, 46). In food preservation they are used to extend the shelf-life and to control pathogens (17). This is achieved by several means. The most common is the decrease of pH to a level where growth of undesirable bacteria is restricted. They also produce a variety of inhibitory substances, including organic acids, hydrogen peroxide, diacetyl, and bacteriocins (see Hurst (21) and Daeschel (11) for reviews). Bacteriocin production has received
considerable attention by dairy researchers in the last decade. Nisin was the first bacteriocin known and used for many years in numerous countries as a food additive to extend the shelf-life or to control specific microorganisms such as clostridia (43), *Listeria monocytogenes* (3) and other Gram-positive pathogens (13, 14, 15, 21).

LAB also have been shown to have a positive impact on human health when consumed as viable cells (probiotic). The linkage of life expectancy to yogurt consumption popularized by Metchnikoff (27) stimulated research in this area. Relevant literature reviews have been written with an emphasis on health roles for lactobacilli and bifidobacteria (33, 35, 36) in humans and/or animals. These microorganisms colonize the intestine and are reported to have different effects. They produce substances inhibitory to pathogenic microorganisms, reduce the cholesterol level in the blood serum of pigs (18, 19, 33), and alleviate lactose intolerance in β-galactosidase-deficient persons (26, 33). They also have been shown to have an anti-cancer effect (7, 33) and to stimulate the immune system (32, 33, 35). For these reasons consumption of fermented dairy products has been advocated by some physicians, scientists and non-scientists. In fact, lactobacilli now are sold in pharmacies and health food stores under a variety of names and to treat a number of illnesses. Today this is an intensive research area and more facts demonstrable in different laboratories are required to substantiate all the health claims.
Since the 1970's, much research has been done on the genetics of LAB. These bacteria have been found to harbor plasmids encoding for important metabolic functions. These plasmids cause genetic instability in lactic acid producing bacteria but also make possible genetic manipulation of the organisms.

The aim of the work presented in this thesis was to study the practical use of LAB to control pathogens (mainly *Listeria monocytogenes*) in some dairy products by their production of bacteriocins as well as to study aroma substances produced by *Leuconostoc*. During the latter study a new selective medium for *Leuconostoc* was developed. Four chapters are presented herein:

Chapter 1: Bacteriocins produced by lactic acid bacteria

Chapter 2: Effect of nisin on growth and survival of *Listeria monocytogenes* in cottage cheese and yogurt.

Chapter 3: Effect of nisin on keeping quality of Feta cheese.

Chapter 4: Development and use of selective medium for isolation of *Leuconostoc* bacteria from vegetables and dairy products.

Research carried out for this thesis was done at three locations. Work on bacteriocins produced by strains of lactic acid bacteria isolated from nature (Chapter 1) was done in Rabat, Morocco at the Institute of Agronomy and Veterinary Medicine, Hassan II; research on the use of the lactococcal bacteriocin nisin (Chapter 2) to inhibit *L.*
monocytogenes in cottage cheese was done at Oregon State University and to inhibit this pathogen in yogurt was done in Morocco; studies on the use of nisin in Feta cheese (Chapter 3) were done at the Federal Dairy Research Center in Kiel, Germany; development of the selective Leuconostoc isolation medium (Chapter 4) was done in Morocco as for Chapter 1.

There is great interest today in the use of natural preservatives to stabilize and protect foods; bacteriocins are the most studied in this regard. The emphasis for this thesis has been to isolate lactic acid bacteria which produce bacteriocins and to examine their possible commercial value. Also, one known bacteriocin (nisin) was used to inhibit a known pathogen (L. monocytogenes) in dairy products. Efforts to isolate naturally-occurring bacteriocin-producing Leuconostoc were thwarted by the unavailability of a useful selective medium. Therefore in the latter phases of this research, such a medium was developed and will find application in future work to isolate the desired Leuconostoc strains.


Chapter 1

Bacteriocins Produced by Lactic Acid Bacteria

Noreddine Benkerroum*

and

William E. Sandine

Department of Microbiology
Oregon State University
Corvallis, OR 97331-3804

Running Title: Bacteriocins

*Present address: Department of Food Microbiology, Institute of Agronomy and Veterinary Medicine, Hassan II, Rabat, Morocco.
ABSTRACT

Lactic acid bacteria (LAB), 324 in number and including 101 identified *Leuconostoc* strains, were screened for bacteriocin production against *Listeria monocytogenes*. Five strains (1.5%) were found to produce antimicrobial substances other than H$_2$O$_2$ or organic acids. Four of these strains were identified as *Lactococcus (Lc.) lactis* and the fifth as a *Lactobacillus (Lb.)* species. Antimicrobial substances produced by *Lc. lactis* strains were confirmed to be protein in nature by their sensitivity to proteases and they had a narrow spectrum of action. Therefore, they were considered to be bacteriocins. Three of these bacteriocins were nisin; the fourth was similar to but distinct from nisin as determined by its different sensitivity to proteases and its spectrum of action. It has been named Lactococcin LB11A. The fifth strain produced an inhibitory substance not inactivated by catalase, not dialysable and with a narrow spectrum of action. It was not protein in nature and therefore not considered to be a bacteriocin.
INTRODUCTION

One of the most important and significant values of LAB to humans is their beneficial role in health. They can act directly when consumed as probiotics (57, 58, 59) or when incorporated in foods such as meat, vegetables and dairy products, or indirectly by action of the antimicrobial substances they produce in foods (8, 16, 24, 38, 39). Several types of antimicrobial substances are produced by various members of this group of microorganisms. The most well known are organic acids, hydrogen peroxide, diacetyl and bacteriocins (8, 24, 38). The latter have received increased research attention during the last decade, these studies focusing mainly on the search for new bacteriocins, their characterization, and purification. An early definition of bacteriocins was given by Jacob et al. (40) when referring to colicins (i.e. the first bacteriocins found to be produced by certain Escherichia coli strains) as proteins that act only on closely related species. Subsequently, a more specific definition was offered by Tagg et al. (65) who stated that for an inhibitory substance to be considered a bacteriocin it must have the following six characteristics: 1) a narrow spectrum of action, 2) bactericidal mode of action, 3) adsorption to specific receptors on cells, 4) production and immunity are plasmid encoded, 5) lethal biosynthesis, 6) has an essential biological moiety for activity. As this definition was quite restrictive and few "bacteriocins" could meet these requirements, the same authors later suggested that bacteriocins produced by Gram positive bacteria should at least be biologically active proteins with a bactericidal mode of action against sensitive microorganisms. Now,
the most widely accepted definition is that "bacteriocins are proteins or protein complexes with a bactericidal mode of action directed against species that are usually closely related to the producer bacterium" (42).

It is now known that bacteriocins are produced by numerous genera of bacteria including those that are Gram-positive, Gram-negative, as well as sporeformers (55). At least four genera of LAB, Lactococcus (32), Leuconostoc (35, 36, 45), Lactobacillus (7, 14, 27, 43) and Pediococcus (13, 37, 50, 62) have been demonstrated to produce a variety of bacteriocins, some of which are rather well characterized. Among these, nisin is the most well known. It is produced by some strains of Lc. lactis subspecies lactis. Nisin has been used for many years in other countries as a food additive to prevent spoilage (16, 20, 21, 24, 39, 54) but only recently has been approved in the United States (26) for addition to processed cheese spread. Intensive research on nisin has been carried out recently, especially to clone the gene encoding for its production (29, 31, 41, 63) and to determine its mode of action on sensitive cells (20). The ultimate goal of bacteriocin research is the legal use of these substances as natural food preservatives.

Among the food-borne pathogens, L. monocytogenes recently has caused health concerns, especially since this microorganism not only survives temperature abuse (23) but grows in refrigerated foods at 2-5°C (11, 57). Therefore, intense research efforts have been made to find means to control the occurrence and survival of this pathogen.
in foods. Nisin was reported to be effective in controlling *Listeria* in cottage cheese (12) and found to control these bacteria in yogurt (Chapter 3 in this thesis). Studies on the sensitivity of *L. monocytogenes* to bacteriocins other than nisin produced by LAB have revealed this pathogen is sensitive to bacteriocins produced by different species of the *Lactobacillus* (1, 36, 45, 61), *Leuconostoc* (35, 36, 45), *Pediococcus* (13, 36, 50, 62), and *Lactococcus* (15, 36, 45, 62) genera.

The purpose of the present study was to survey LAB isolated from raw milk and different dairy products produced in Morocco for the production of bacteriocins and to characterize the producer bacteria as well as the inhibitory substance produced.
MATERIALS AND METHODS

Bacterial Strains and Media:

Bacteria tested for bacteriocin production were isolated from raw milk, Lben\(^{(1)}\), raw butter\(^{(2)}\), bakery yeast and pickle brine. Except for the butter, products were serially diluted in 0.85% saline and 0.1-ml aliquots were surface spread plated on M17 (66), MRS (22) and Elliker (25) agar media and then incubated for 24 hours at 30°C. Butter was melted at 40°C and centrifuged at 4000 rpm for 10 min in a Heraeus Sepatech centrifuge. The aqueous phase was serially diluted and surface plated as described above. After incubation, colonies were randomly selected, grown in MRS broth, Gram stained and tested for catalase production. Gram negative and/or catalase positive strains were discarded. Those to be studied were grown and frozen at -20°C in sterile 10% reconstituted nonfat dry milk. Working cultures were transferred to MRS broth and slants of MRS agar for short time storage. All incubations were at 30°C, unless otherwise indicated.

In addition to the above-described isolates, 101 Leuconostoc spp. isolated from raw milk and vegetables on a newly developed Leuconostoc selective medium formulated during the present research were tested for bacteriocin production. These strains were identified as Leuconostoc according to tests recommended by Garvie (30).

\(^{(1)}\) Lben: A traditional Moroccan raw fermented milk, soured spontaneously and then churned to separate the Lben from the butter\(^{(2)}\).
Indicator organisms used in this study (Table 1.1) were maintained as stab cultures on TSA (Biokar) or MRS agar, at 40°C and transferred weekly. Prior to use they were propagated in appropriate broth media.

**Detection of Bacteriocinogenic Strains:**

Bacteriocin producers were screened against *L. monocytogenes* using the spot and the well diffusion methods as described by Spelhaug and Harlander (62) and Tagg and McGiven, (64), respectively. In the spot technique, an overnight culture of the test organism grown in MRS broth supplemented with 2.5% yeast extract and 2% glucose (MRSYG) was diluted 10 fold in 10 mM Tris HCl (pH 7.0) and 2 µl aliquots were spotted onto M17 and MRS agar. Inoculated plates were incubated until growth was evident, then overlaid with 5 ml of TS soft agar (0.7% agar) seeded with 0.1-ml of an overnight culture of *L. monocytogenes*. Plates were incubated for an additional 18 hours then checked for clear zones around spots of the putative producers.

In the well diffusion assay, 20 ml of Muller Hinton agar (Biokar), or MRS soft agar in the case of LAB, were seeded with 0.1 ml of an overnight culture of the indicator organism, poured in a sterile Petri dish and allowed to harden. Plates were then put into the refrigerator for 30 min to 1 hr. Wells, 8mm in diameter, were punched in the medium using stainless steel tubing and then they were filled with 60 µl of the test culture supernatant. The
supernatant was prepared by growing the test organisms in MRSYG broth for 24 hours; one ml of the culture was then transferred to a 1.5-ml sterile Eppendorf tube and centrifuged in a microfuge (Fisher Scientific, Model 235C). At this point, filter sterilization of the supernatant was avoided since it has been shown that some bacteriocins are retained on filter surfaces (52).

Strains selected for further study were identified by using the following tests: ammonia production from arginine, CO₂ production from glucose in citrate-supplemented (0.2%) milk, dextran production, growth at different temperatures (10, 43 and 44°C), milk coagulation within 16 hours at 22°C (fast cultures) and litmus reduction.

Elimination of Organic Acids, Hydrogen Peroxide and Bacteriophages as Inhibitory Agents:

The effect of organic acids was ruled out by adjusting the pH of the test clear supernatant to 6.0 with 10 M NaOH which was then filter sterilized with a 0.22-μl Millipore filter membrane and tested by the well diffusion assay for the persistence of the inhibition zone.

To exclude the effect of hydrogen peroxide, catalase (EC1.11.1.6, Sigma Chemical Co.) was used in two ways:

1. Incorporation in the overlay agar, for the spot test, to a final concentration of 68 IU/ml as described by Barefoot and Klaenhammer (10).
2. Treatment of the cell-free supernatant with 650 IU/ml of catalase in Tris HCl buffer (pH 8.0) for 2 hours at 37°C. Controls were; Tris HCl buffer with and without enzyme, and non-treated cell free supernatant to which has been added the same quantity of buffer as in the reaction mixture. After treatment, activity was assayed by the well diffusion method.

Evidence that the inhibitory action was not due to a bacteriophage was obtained in two ways:

1. A lawn of the indicator strain was streaked with an inoculating loop, which has been stabbed into the zone of inhibition surrounding the well, according to McCormick and Savage (47). The plate was incubated until growth was evident and then examined for plaque formation along the streak line.

2. Phage titer of a 24-hour culture of the test organisms was determined as described by Terzaghi and Sandine (66).

Mode of Action:

A modification of the cell diffusion assay was used. Muller Hinton agar (20 ml) was seeded with 0.1-ml of a sensitive indicator culture, poured in a sterile Petri dish and allowed to harden. It was incubated until growth was evident. Then wells were cut and filled with 60 μl of cell free supernatant. Incubation of plates was continued and then they were examined for clearing around the wells at different time intervals starting from 3 hours. Clearing indicated cell lysis.
Another procedure to test for bactericidal action was used. M17 agar (20 ml) supplemented with glucose (0.5%) and seeded with 0.1-ml of a sensitive indicator culture was incubated as in the first case until growth was evident. The test culture was then streaked onto this plate and incubation continued for an additional 24 to 48 hours and then checked for clearing around the streak line.

In addition to these techniques, and for confirmation, growth of the indicator strain in neutralized (pH 7) cell-free supernatant (CFS) was monitored by following the optical density at 600 nm during incubation. The CFS was inoculated with 0.1-ml of an overnight *L. monocytogenes* culture and incubated. Sterile MRS broth (10-ml) was similarly inoculated with the indicator organism and incubated to serve as a control. Samples of 1 ml each were aseptically removed at 0, 2, 4, 7, 9, and 24 hours and their optical densities determined at 600 nm by using a Jenway PC01 Colorimeter.

**Action of Enzymes:**

The cell free supernatants of possible bacteriocin producers adjusted to pH 6 were treated separately with 6 different enzymes. Each enzyme was obtained from Sigma Chemical Company except trypsin, which was from Serva: trypsin (EC 3.4.21.4), α-chymotrypsin (EC 3.4.21.1), pronase E (Type XXV), RNase (EC 3.1.27.6), lysozyme (EC 3.2.1.17) and lipase (Type XIII). Trypsin, α-chymotrypsin, pronase and lipase type XIII were dissolved in phosphate buffer (0.1 M, pH 6). RNase was in 0.05 M Tris 15 mM NaCl, (pH 8) and lysozyme in 25
mM Tris (pH 8). Enzyme solutions were mixed with cell free supernatants (1:1) to a final concentration of 1 mg/ml. Incubation was at 37°C in a water bath for 2 hours; then samples were boiled for 3 min to stop the reactions. This treatment did not terminate the RNase reaction, which continued during the experiment. Controls included sterile MRS broth, a 1:1 mixture of culture supernatant with buffer by without enzyme and buffer with only the enzyme. After boiling, each sample was assayed for bacteriocin activity by the well diffusion method.

Nisin (Alpin and Barrett, Trowbridge, England) to a final concentration of 36 x 10² IU/ml was used along with the CFS for comparison purposes.

Resistance to chloroform and heat:

Chloroform was thoroughly mixed with the producer culture (1:10) and left at room temperature (25°C) for 1 hour. The mixture was then centrifuged at 5000 rpm; chloroform was removed and activity in the aqueous phase assayed by the well diffusion method.

Aliquots of cell free supernatants also were dispensed in different test tubes and heated at 60°C for 30 min, 80°C for 30 min, 100°C for 10 min or autoclaved (121°C for 15 min). The samples were cooled and assayed for activity. Unheated cell free supernatants were used as positive controls.
Testing the effect of heat at various pH levels was accomplished by using two series of test tubes, each containing 4 ml of clear culture supernatant. In each series, the pH of the supernatant was adjusted to 2, 3, 4, 4.5, 5.0, 6.0, 7.0, 8.0, 9.0, 10, 11 or 12 with concentrated HCl or 10 M NaOH. After pH adjustment, one series was boiled for 10 min. Supernatant from a non-producer strain was treated in the same way but was not heated and was used as a negative control. After treatment, activity was assayed against *L. monocytogenes*.

**Spectrum of Action:**

The spectrum of activity against different bacteria was determined by the spot technique (62) using M17 agar as a bottom layer and MRS soft agar as the top layer when the indicator strains were a LAB or soft TSA for other indicator microorganisms. Those used as indicators are listed in Table 1.1. Producer microorganisms were also tested against each other and against themselves.

**Dynamics of Bacteriocin Production:**

Production of bacteriocins was monitored during growth of producer strains at 30°C in MRSYG broth by determination of arbitrary units (AU) after 2, 4, 5, 7, 10, 24 and 48 hours of incubation. Reciprocal of the highest dilution showing a definite inhibition of an indicator strain in the well diffusion assay was expressed as an AU per 60 ml. The pH of the culture as well as the OD at 600 nm were recorded at the same intervals.
Associative Growth:

The effect of bacteriocin producers on growth of *L. monocytogenes* in mixed culture was assessed in MRSYG and in 10% reconstituted nonfat dry milk. Sterile MRSYG broth (20 ml) and 10% NDM autoclaved at 121°C for 10 min were simultaneously inoculated with 0.1 ml of an overnight culture of *L. monocytogenes* and the producer strain. They were then incubated and numbers of *L. monocytogenes* determined on ASL medium (3) modified by omission of moxalactam. Samples were taken at 0.5, 7, 9, 24 and 48 hours. Controls included 10% NDM and MRSYG inoculated with *L. monocytogenes*, as well as 10% NDM and MRSYG inoculated with potential producer organisms and sterile 10% NDM.

Plasmid Isolation:

A modified small scale lysis procedure described by Anderson and McKay (6) was used to isolate plasmid DNA. Lysis medium was MRS supplemented with 20 mM DL threonine (Sigma) for LAB and TSB for *E. coli* V517. Strains were grown at 30°C for 4 to 5 hrs and cells harvested by centrifugation at 5,000 rpm (Heraeus Sepatech) for 10 min at 25°C. Washed cells were resuspended in 379 µl of TES buffer (67% sucrose, 50 mM Tris, 1 mM EDTA, pH 8) and transferred to a 1.5 ml Eppendorf tube and then 96 µl of lysis buffer (20 mg/ml lysozyme (Sigma) in 0.05M Tris, pH 8.0) was added. After 30 min of incubation at 37°C, 27.6 µl of SDS solution (20% [w/v] in 50 mM Tris, 20 mM EDTA, pH 8.0) was added to complete lysis. The DNA was denatured with 27.6 µl of 3M NaOH, freshly prepared, followed by
gentle mixing for 5 min; then the plasmid DNA was renatured by neutralization with 45.6 µl of 2M Tris (pH 7.0) followed by gentle mixing for 5 min. Precipitation of chromosomal DNA was achieved by addition of 71.7 µl of 5 M NaCl and overnight incubation at 4°C. Samples were then centrifuged in a Fisher Scientific microfuge at maximum speed for 20 min. Supernatants were collected in centrifuge tubes and extracted successively with 700 µl of distilled phenol saturated with 3% NaCl and 700 µl chloroform: isoamyl alcohol (24:1). Plasmid DNA was then precipitated in two volumes of cold ethanol at 4°C for 1.5 hours and centrifuged for 15 mins at maximum speed. Plasmid pellets were dried for 15 min and resuspended in 10 µl of TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8). Five ml of RNase solution (10 mg/ml) was added to plasmid DNA samples, which were then incubated at 37°C for 15 min.

**Agarose Gel Electrophoresis:**

Electrophoresis was performed in a Photodyne apparatus according to the manufacturer's recommendations. Agarose concentration was 0.7% (Agarose Type II, Sigma). Gel was run in TAE buffer at 8.0 V/cm on 12 to 15 µl DNA lysates to which had been added 5 µl of tracking dye. *E. coli* V517 plasmids were used as standards to estimate the molecular weight of unknown plasmids.
Curing Trials:

Attempts were made to cure putative bacteriocin-producing strains by two methods: growth at sublethal temperature and exposure to curing agents. Strains were grown in MRS broth and subcultured daily for up to 10 days at 43 C using a 1% inoculum. After exposure to a curing agent, 1 ml of an overnight culture was inoculated into 10 ml of MRS broth containing 25 μg/ml of ethidium bromide (Sigma) and incubated. Test tubes were covered with aluminum foil to protect ethidium bromide from light. After 3 and 7 days of incubation, serial dilutions were made and 0.1-ml aliquots of appropriate dilutions were surface plated on MRS agar and incubated. Individual colonies were selected, grown in MRSYG broth and tested for bacteriocin production by the well diffusion assay.
RESULTS

Screening of LAB for Inhibition of *L. monocytogenes*:

Strains isolated from different plant and food products, 324 in number, were tested for antibiosis against *L. monocytogenes* (Table 1.2). Eighty-seven (26.9%) gave positive results in the well diffusion assay when the pH of the supernatant was unchanged. However, most of the inhibition zones were either small (diameter less than 12 mm including the well) or hazy. When the pH of the supernatant was adjusted to 6, only 4 strains (1.2%) retained their activity. These strains plus another one (1.5%) were positive in the spot test with M17G agar as the bottom layer. When MRS agar was used as a bottom layer, similar results to those of the well diffusion assay using non-neutralized supernatant were obtained (i.e., 87 strains positive).

The five strains found positive in the spot test, including the four positive in the well diffusion assay with pH of the supernatant adjusted to 6, were identified by physiological and morphological tests classically used for LAB identification (Table 1.3). Four strains were identified to the species level as *Lactococcus lactis* and one was identified to the genus level as a *Lactobacillus* species (Table 1.4).

Nature of Inhibitor Substances:

Substances produced by the five selected strains were not hydrogen peroxide, bacteriophages or organic acids. The inhibitory activity
was not affected by catalase and no plaques were formed when an inoculating loop was stabbed in the zones of inhibition and streaked on a lawn of \textit{L. monocytogenes}. Also, phage titer determination of producer strain cell free supernatants were negative. As for organic acids, despite the fact that one strain, Y5, showed no activity at pH 6, the substance that it produced was not dialysable (Visking tubing 8000 molecular weight cutoff, Serva), but retained activity at pH 4.6, suggesting that it was not an organic acid. All substances were insensitive to ribonuclease, lysozyme and lipase. Four culture activities were sensitive to proteases, but the fifth was not tested in this regard (Table 1.5). Three out of the four tested had the same sensitivity pattern to enzymes as nisin, while the fourth (LBII) differed from nisin in that it was sensitive to trypsin and \(\alpha\)-chymotrypsin.

**Properties of Inhibitory Substances:**

Table 1.6 summarizes properties of the inhibitory substances. They were retained by a membrane tubing with a molecular weight cutoff of 8000 when dialysed against polyethylene glycol 8000 (Sigma). They were stable to the different heat treatments and to chloroform. As the substances produced by S29, S41 and S60 behaved like nisin, their resistance to boiling at pH 2 and pH 1.1 was examined. These results, as well as their range of inhibition against various bacteria, (data not shown) indicated that these substances were nisin. Therefore strains producing them were discarded. Further study was focused on the LBII strain because it was very inhibitory to \textit{L.}
monocytogenes and the bacteriocin produced was elaborated by a GRAS (Generally Recognized as Safe) bacterium, Lc. lactis.

Stability of LBII and Y5 Antimicrobial Substances to pH and Combined pH and Heat:

Both substances were more active at pH values less than 4 (Table 1.7). Y5 activity gradually diminished from pH 2 to pH 5 in both heated and unheated samples. The non-producer strain used as a negative control did not show any inhibition zone above pH 3 or below pH 12. Small turbid zones could be observed, suggesting that the large and clear zones produced by Y5 at pH values below 4.6 was not due to acidity alone and that the low pH was a stimulatory factor. Heated and unheated samples acted in the same way. As for the LBII inhibitory substance, its activity also decreased as the pH increased but it was completely lost at pH values above 10 when samples were not heated and at pH values higher than 8 when they were heated at 100°C for 10 min (Figure 1.1). These data indicated that LBII was behaving like nisin; however, when the pH of LBII supernatants was adjusted to 11 and held at room temperature for one hour and then readjusted to 7, activity was totally recovered. Nisin activity was irreversibly lost with this treatment, suggesting that LBII produced a bacteriocin different from nisin.
**Bactericidal Mode of Action:**

The bactericidal mode of action was demonstrated by the well diffusion and streak assays against *L. monocytogenes* and *Streptococcus* sp. Also, when *L. monocytogenes* was grown in *Lc. lactis* LB11 CFS at pH 7.0, the optical density decreased to a very low level (Figure 1.2). *L. monocytogenes* grew well in spent broth from a culture of a non-producer strain at pH 7.0 which was used as a positive control (data not shown). However, since spent broth from the producer strain, treated with protease to inactivate the bacteriocin, was not used as a positive control, the best evidence presented herein for the bactericidal mode of action remains as described. As for Y5, 10-fold concentration of the CFS was necessary to demonstrate the bactericidal mode of action by the well diffusion assay, however, it was demonstrated in mixed culture growth with *L. monocytogenes* (Figure 1.5).

**Spectrum of Action:**

Table 1.8 shows the range of activity of LBII and Y5 bacteriocins against different bacterial strains. LBII and Y5 did not inhibit each other and, as expected, did not inhibit themselves. LBII was more active on Gram positive than on Gram negative bacteria, although it inhibited *E. coli* V517 and *Salmonella typhimurium* to some extent. Its spectrum of action was similar to nisin, the main difference being that it did not inhibit *Micrococcus flavus* which is very sensitive to nisin and is even used for nisin quantitation in foods, according to
Fowler et al. (28). On the other hand, Y5 had a very narrow spectrum of action. The most sensitive strains to it were *Streptococcus* sp. and *Salmonella typhimurium*, but it also inhibited *Lactococcus lactis* 7962, a nisin producer. Inhibition zones of Y5 on sensitive indicators were improved when plates were incubated 48 hours before adding the indicator-seeded top layer or by using MRS agar as a bottom layer instead of M17G.

**Dynamics of LBII Inhibitory Substance (IS) Production:**

Figure 1.3 summarizes results on the production of LBII IS. Detection of this substance by the well diffusion assay starts after 5 hours of incubation and reaches its maximum between 10 and 24 hours. After 7 hours of incubation, 30% of the bacteriocin is produced when the pH is 4.6 and the cell growth is maximum. Therefore 70% of activity units are produced during the stationary phase, indicating that LBIII IS may be a secondary metabolite as is the case for many known bacteriocins such as Pediocin AcH (13) and nisin (39).

**Associative Growth:**

Associative growth of *L. monocytogenes* with *Lc. lactis* strains LBII and Y5 was examined in 10% reconstituted NDM and in MRYG (Figure 1.4 and Figure 1.5, respectively). The *L. monocytogenes* counts were higher than $10^8$ in both media after 24 hours of incubation when grown alone. However, when in association with LBII, growth was
reduced about 4 log units after 9 hours of incubation in MRSYG and in nonfat milk as well. No significant difference (P <0.001) occurred in reduction of the growth rate of L. monocytogenes when comparing MRSYG and reconstituted milk. As for growth of L. monocytogenes with strain Y5 (Figure 1.5), a significant decrease in Listeria counts could be observed only after 24 hours in MRSYG and after 48 hours in nonfat milk. The pathogen was killed by Y5 within 48 hours in MRSYG and within 72 hours in milk. Such a difference can have two explanations: The first is that MRSYG is a better medium for production of the inhibitory substance than milk. Glucose and yeast extract were shown to have a stimulatory effect on bacteriocin production (13). The second could be the fact that Y5 IS has a maximum activity at low pH (Table 1.7). In fact, the pH dropped to about 3.7 in MRSYG after 24 hours while it took 48 hours to reach that pH in milk (data not shown). The killing of L. monocytogenes in the mixed culture with Y5 also indicates a bactericidal effect, however, at this low pH it is difficult to tell whether it is the acidity or IS activity that plays the major antibiosis role. It is likely that there is a synergistic action between the acidity and the inhibitory activity of the substance. This is rather common as shown by Raccah et al. (51). In fact, lactostrepsins produced by lactococci behave in a similar way and thus have been named "acid bacteriocins". Furthermore, Abdel-bar et al. (1) showed that Lb. bulgaricus produced a non-proteinoaceous antimicrobial substance which is active only at pH 4.0 and below.
LBII Possibly Produces More than One Bacteriocin:

Two observations led us to conclude that LBII produces more than one bacteriocin: First, after protease treatment, activity was totally lost (Table 1.9) on L. monocytogenes but only slightly reduced on the hemolytic Streptococcus sp. Second, after curing, some mutants lost their activity against L. monocytogenes but still inhibited the Streptococcus sp. Strains producing more than one bacteriocin have been described previously (2).

Plasmid Profile of Producer Strains:

The plasmid profiles of Lc. lactis LBII and Lactobacillus sp. Y5 and their mutants are shown in Figures 1.6 and 1.7, respectively. These plasmid profiles were consistently observed in repeated trials. Their estimated molecular weights are shown in Table 1.10. Both wild type strains harbor plasmids with MW ranging from 1.8 mDa to 30 mDa in the case of Y5, and from 4.9 mDa to 50 mDa in the case of LBII. The occurrence of multiple plasmids in these organisms is not unusual.

Curing of Y5 by incubation at sublethal temperatures (43°C) gave mutants lacking the 5.2 mDa plasmid (Figure 1.7, Lines c, d, and e) without concomitant loss of IS production. LBII, however, gave 10 mutants not producing the IS against L. monocytogenes, among which 5 were still inhibitory to the Streptococcus sp. After a second transfer the 5 mutants regained their capability to inhibit L. monocytogenes. All LBII mutants showed a strikingly different
plasmid profile from the parental strain, while their morphological and physiological characteristics remained the same. The mutants had more plasmids than the wild type (Figure 1.6); some of the extra plasmids migrated further than the smallest parental plasmid and cannot therefore be considered as open forms.
DISCUSSION

Results of this study show that antibiosis among LAB is common; 27% of the 324 isolates were inhibitory to *L. monocytogenes* as determined by the well diffusion assay. However, the majority of substances were inactive at pH 6, suggesting that organic acids are common metabolites used by this group of organisms to antagonize other microorganisms. Less common, however, is the production of bacteriocins (32, 36, 62). In this study, 4 strains (1.2% of those tested) were bacteriocin producers while one is still to be confirmed. Geis et al. (32) made a survey on 280 lactic streptococci (lactococci) for bacteriocin production and found 23% producing antimicrobial substances by direct methods; 6% were confirmed to produce bacteriocins. The higher frequency of bacteriocin production found in their work compared to this work may be explained by the fact that these workers used four indicator strains related to the test organism, which greatly increases the chances to detect bacteriocinogenic strains. In the present study it was also shown that the assay technique as well as the media used for screening have a great impact on the results. Such has been also shown by Spelhaug and Harlander (62). The well diffusion assay with the pH of the test supernatant adjusted to 6 is widely used for this purpose, but it has two major limitations that lead to false negative results: 1) diffusion of the substance may be impeded (19), 2) "acid bacteriocins" would not be detected. As for the spot test, media used as top and bottom layers must be chosen with care. In our hands, when MRS agar was used as a bottom layer, false positive results
were obtained, while M17G agar gave better results and would be appropriate for such studies. These findings are in perfect agreement with those obtained by Spelhaug and Harlander (62). Nonetheless, MRS or MRS fortified with glucose and yeast extract was reported to be suitable medium for bacteriocin production by LAB (32). Therefore MRS or MRSYG are recommended for any kind of study on the production of bacteriocins but the screening.

Among the 5 producers we found, 4 were *Lc. lactis* and one was a *Lactobacillus* species. No *Leuconostoc* were found to be inhibitory against *L. monocytogenes*, although 101 identified *Leuconostoc* strains were examined. The production of bacteriocins by *Leuconostoc* seems not to be a common phenomenon. Few studies, to our knowledge, have described the production of antimicrobial substances by this group. Of the four lactococci found to produce inhibitory substances, three were producing nisin. These results suggest that bacteriocins against *L. monocytogenes* are more frequently produced by *Lc. lactis* and that strains of this species mostly produce nisin, but not exclusively.

The *Lc. lactis* LBII strain appeared to produce two inhibitory substances. The first, active against *L. monocytogenes*, was inactivated by trypsin, α-chymotrypsin and pronase, and the second, inhibitory for *Streptococcus* sp., was not destroyed by α-chymotrypsin. The former received the most attention in this study. Production of more than one inhibitory substance by the same strain has been reported previously (2). The LBII inhibitor, which was
inactivated by the three proteases, fits the definition of a bacteriocin as given by Klaenhammer (42). Therefore we considered it as a bacteriocin and propose the name of Lactococcin LBIIA. This substance shares many properties with nisin but differs from it by two main characteristics: spectrum of action and sensitivity to proteases, namely to trypsin and α-chymotrypsin, to which nisin is resistant. It needs to be pointed out, however, that there are conflicting data about sensitivity of nisin to α-chymotrypsin; some authors found it sensitive (38, 39) while others demonstrated its resistance (16). In our case the nisin used (Applin & Barrett) was resistant; nonetheless, there is a general agreement on resistance of nisin to trypsin. The production of substances different from nisin by strains of Lc. lactis has been reported by other workers. Geis et al. (32) separated the inhibitory substances produced by L. lactis strains into 3 types on the basis of their inhibitory spectra and chemical properties. Lactococcin LBIIA would not fit in any of these types. It is similar to Type VI bacteriocins, but according to these authors substances of this type are resistant to trypsin which is not the case for our substance. Carminati et al. (15) found 7 strains of Lc. lactis producing inhibitory substances that differed from nisin in their sensitivity to proteases. The present investigation involved use of fewer proteases and therefore, it is difficult to compare Lactococcin LBIIA to those studied by Carminati et al. (15) Bacteriocin production was shown to be either plasmid (2, 15, 17, 18, 33, 34, 37, 48, 53, 60) or chromosomally (47, 49) encoded. Mutation studies done to relate lactococcin LBII production to plasmids were
not conclusive; instead they raised intriguing questions that we could not answer (Figure 1.6).

Y5 is a *Lactobacillus* strain that produces a non-dialyzable inhibitory substance, not destroyed by catalase, suggesting that it is neither an organic acid nor hydrogen peroxide. It also is not a bacteriophage but it has a bactericidal effect. Nonetheless, this substance could not be considered a bacteriocin or a bacteriocin-like substance since its protein nature was not demonstrated in this study. Proteases we used were all active at a neutral pH, where the inhibitory substance produced by Y5 was inactivated. If we rely on the accuracy of the spot technique using M17G as a bottom layer for detecting bacteriocins, this substance would be similar to lactostrepsins (9, 45) or to Microgard™ (Wesman Foods, Inc., Beaverton, Oregon). The latter, presumably a bacteriocin (5) and produced by *Propionibacterium shermanii*, shows similar activity with regard to pH as reported by Al-Zoreky et al.(4). Further characterization of the substance produced by *Lactobacillus* Y5 is being carried out.
Table 1.1: Indicator bacteria, their origin and culture media used for their growth.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin</th>
<th>Medium(^{(1)})</th>
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<tbody>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>ATCC</td>
<td>TS(^{(2)})</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>IAVHII</td>
<td>TS</td>
</tr>
<tr>
<td><em>Escherichia coli V517</em></td>
<td>OSU</td>
<td>TS</td>
</tr>
<tr>
<td><em>Streptococcus sp.</em></td>
<td>IAVHII</td>
<td>TS</td>
</tr>
<tr>
<td><em>Micrococcus flavus NCIB8166</em></td>
<td>OSU</td>
<td>TS</td>
</tr>
<tr>
<td><em>Leu. dextranicum 187</em></td>
<td>OSU</td>
<td>MRS</td>
</tr>
<tr>
<td><em>Leu. cremoris</em></td>
<td>OSU</td>
<td>MRS</td>
</tr>
<tr>
<td><em>Leu. cremoris dM711</em></td>
<td>FDRC</td>
<td>MRS</td>
</tr>
<tr>
<td><em>Lactococcus lactis ssp. lactis</em> biovar.diacetylactis BU2</td>
<td>FDRC</td>
<td>MRS</td>
</tr>
<tr>
<td><em>Lc. lactis 7962</em></td>
<td>OSU</td>
<td>MRS</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td>IAVHII</td>
<td>TS</td>
</tr>
</tbody>
</table>

OSU = Oregon State University (Corvallis, USA)
IAVHII = Institut Agronomique et Veterinaire Hassan II Rabat, Morocco
FDRC = Federal Dairy Research Center (Kiel, Germany)

\(^{(1)}\)media were broth in the case of propagation and agar slants for storage.

\(^{(2)}\)TS - Trypticase Soy.
Table 1.2: Number and percentage of strains of LAB antagonizing growth of *L. monocytogenes*

<table>
<thead>
<tr>
<th>No. of strains</th>
<th>Source</th>
<th>Well diffusion assay</th>
<th>Spot test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Supernatant&lt;sup&gt;1&lt;/sup&gt;</td>
<td>N. CFS&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>22</td>
<td>Raw butter</td>
<td>12(54%)</td>
<td>0</td>
</tr>
<tr>
<td>43</td>
<td>Lben</td>
<td>19(44%)</td>
<td>1(2.3%)</td>
</tr>
<tr>
<td>83</td>
<td>raw milk</td>
<td>17(40%)</td>
<td>1(1.2%)</td>
</tr>
<tr>
<td>67</td>
<td>Bakery Yeast</td>
<td>78(42%)</td>
<td>2(2.9%)</td>
</tr>
<tr>
<td>101</td>
<td>Mix. of Vegetable&lt;sup&gt;3&lt;/sup&gt;</td>
<td>5(5%)</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>Pickle brine</td>
<td>6(75%)</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>1</sup>Used without pH adjustment

<sup>2</sup>Neutralized cell-free supernatant (pH 6)

<sup>3</sup>All *Leuconostoc* species
Table 1.3: Morphological and physiological tests used for bacteriocin producer strain identification.

<table>
<thead>
<tr>
<th>Test</th>
<th>S29</th>
<th>S60</th>
<th>S49</th>
<th>Y5</th>
<th>LBII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram reaction</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Morphology</td>
<td>c</td>
<td>c</td>
<td>c</td>
<td>r</td>
<td>c</td>
</tr>
<tr>
<td>Catalase production</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gas from glucose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gas from citrate</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dextran production</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Growth at 10°C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Growth at 42°C</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth at 44°C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Milk coagulation</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Litmus reduction</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Arginine deamination</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Table 1.4: Identification of bacteriocin-producing strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>S29</td>
<td>Bakery yeast</td>
<td><em>Lactococcus lactis</em></td>
</tr>
<tr>
<td>S41</td>
<td>Raw milk</td>
<td><em>Lactococcus lactis</em></td>
</tr>
<tr>
<td>S60</td>
<td>Bakery yeast</td>
<td><em>Lactococcus lactis</em></td>
</tr>
<tr>
<td>Y5</td>
<td>Lben</td>
<td><em>Lactobacillus</em> species</td>
</tr>
<tr>
<td>LBII</td>
<td>Lben</td>
<td><em>Lactococcus lactis</em></td>
</tr>
</tbody>
</table>
Table 1.5: Effect of various enzymes on the inhibitory activity of lactic acid bacterial strains against *L. monocytogenes*.

<table>
<thead>
<tr>
<th>Strain</th>
<th>α-Chym</th>
<th>Trypsin</th>
<th>Pronase</th>
<th>Lipase</th>
<th>Lyzosyme</th>
<th>Ribonuclease</th>
</tr>
</thead>
<tbody>
<tr>
<td>S29</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>S41</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>S60</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>LBII</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Nisin</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
</tbody>
</table>

R = resistant
S = sensitive
Table 1.6: Stability of inhibitory substances to different treatments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Inhibitory substance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat</td>
<td>S29  S60  S41  Y5  LBII</td>
</tr>
<tr>
<td>100°C for 10 min</td>
<td>R  R  R  R  R  R</td>
</tr>
<tr>
<td>60°C for 30 min</td>
<td>R  R  R  R  R  R</td>
</tr>
<tr>
<td>80°C for 30 min</td>
<td>R  R  R  R  R  R</td>
</tr>
<tr>
<td>Autoclaving (121°C, 15 min)</td>
<td>R  R  R  R  R  R</td>
</tr>
<tr>
<td>Chloroform</td>
<td>R  R  R  R  R  R</td>
</tr>
<tr>
<td>Dialysis</td>
<td>Ret  Ret  Ret  Ret  Ret</td>
</tr>
<tr>
<td>Filtration</td>
<td>P  P  P  P  P  P</td>
</tr>
</tbody>
</table>

R = Resistant
Ret = Retained
P = Passes
Table 1.7: Stability of LBII and Y5 inhibitory substances to pH and heat.

<table>
<thead>
<tr>
<th>pH</th>
<th>LBII Unheated Samples</th>
<th>LBII Heated Samples</th>
<th>Y5 Unheated Samples</th>
<th>Y5 Heated Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>18</td>
<td>16</td>
<td>15.00</td>
<td>15.00</td>
</tr>
<tr>
<td>3</td>
<td>ND</td>
<td>ND</td>
<td>14.00</td>
<td>14.00</td>
</tr>
<tr>
<td>4</td>
<td>17</td>
<td>14.5</td>
<td>13.00</td>
<td>13.00</td>
</tr>
<tr>
<td>4.6</td>
<td>ND</td>
<td>ND</td>
<td>11.00</td>
<td>11.00</td>
</tr>
<tr>
<td>5.0</td>
<td>17</td>
<td>14.0</td>
<td>10.50</td>
<td>10.00</td>
</tr>
<tr>
<td>6.0</td>
<td>15.0</td>
<td>13.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7.0</td>
<td>15</td>
<td>13.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8.0</td>
<td>14.5</td>
<td>10.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9.0</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10.0</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>11.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* diameter of inhibition zones include the well diameter (8mm) except when it was zero
ND = not determined
Data are average values of two determinations, each one in duplicate.
Table 1.8: Inhibitory activity of LBII and Y5 against Gram positive bacteria and *E. coli*.

<table>
<thead>
<tr>
<th>Indicator Strain</th>
<th>LBII</th>
<th>Y5</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Micrococcus flavus</em></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td><em>E. coli V517</em></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>Streptococcus</em> sp.</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td><em>Lc. diacetylactis</em> BU2</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td><em>Leu. cremoris</em> 44-4C</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td><em>Leu. dextranicum</em> 181</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td><em>Lc. lactis Y5</em></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Lc. lactis LBII</em></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Leu. cremoris</em> M711</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Lc. diacetylactis</em> F7122</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td><em>Lc. lactis</em> 7962</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td><em>L. monocytogenes</em></td>
<td>+++</td>
<td>+</td>
</tr>
</tbody>
</table>

- : No zone of inhibition  
+: Diameter ≤ spot  
++: Diameter ≥ 2 x spot diameter  
+++: < 2 x spot diameter
Table 1.9: Activity assay of *Lc. lactis* LBII bacteriocin after treatment with α-chymotrypsin on *L. monocytogenes* and *Streptococcus* sp.

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Treated CFS</th>
<th>CFS</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. monocytogenes</em></td>
<td>0</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td><em>Streptococcus</em> sp.</td>
<td>12</td>
<td>15</td>
<td>0</td>
</tr>
</tbody>
</table>

CFS = cell-free supernatant
Table 1.10: Plasmid content of the producer strains (*Lactobacillus* sp. Y5 and *Lactococcus lactis* LBII) and their derivatives after curing trials.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Production of Inhibitory Substance(^1)</th>
<th>Plasmid Content (mDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y5</td>
<td>+</td>
<td>1.8; 3.7; 5.2; 6.8; 8.6; 9.6; 21; 30</td>
</tr>
<tr>
<td>Y5(d)2</td>
<td>+</td>
<td>1.8; 3.7; 6.8; 8.6; 9.6; 21; 30</td>
</tr>
<tr>
<td>LBII</td>
<td>+</td>
<td>4.9; 5.4; 9.0; 21; 38; 50</td>
</tr>
<tr>
<td>LBII(d1)</td>
<td>-</td>
<td>2.2; 9.0; 27; 30; 43</td>
</tr>
<tr>
<td>LBII(d2)</td>
<td>-</td>
<td>2.2; 3.7; 8.4; 9.0; 27; 30; 43</td>
</tr>
</tbody>
</table>

\(^1\)+ produces the IS
\(^2\)All mutants had the same plasmid profiles but only one is shown on this table.
Figure 1.1: Effect of pH and heat on the antimicrobial substance produced by *Lactococcus lactis* LBII.

**Right:** Samples heated at 100°C for 10 min. before testing. Clockwise from the top:
- Cell free supernatant (CFS), pH 10
- CFS, pH 8
- CFS, pH 6
- CFS, pH 4
- MRS (sterile)
- CFS, pH 11

**Left:** Non-heated samples. Clockwise from the top:
- Cell free supernatant (CFS), pH 10
- CFS, pH 8
- CFS, pH 6
- CFS, pH 4
- MRS (sterile)
- CFS, pH 11
Figure 1.2: Growth of *Listeria monocytogenes* (O.D. at 600 nm) in MRS broth and in *Lactococcus lactis* LBII cell-free-supernatant.
Figure 1.3: Dynamics of LBII Inhibitory Substance (IS) production as a function of time, pH and growth (O.D.)
Figure 1.4: Associative growth of *Listeria monocytogenes (L.m.)* and *Lactococcus lactis* LBII in MRSYG and in reconstituted non-fat dry milk (NDM).
Figure 1.5: Associative growth of *Listeria monocytogenes* (*L.m*) and *Lactobacillus* sp Y5 in MRSYG and in reconstituted non-fat dry milk (NDM).
Figure 1.6: Plasmid patterns of *lactococcus lactis* LBII and its derivatives after curing trials with 25 µg/ml of ethidium bromide.

Lane A: Standard; *E. coli* V517  
Lane B: Parental Strain (Lc. Lactis LBII)  
Lane C & D: LBII(d1) and LBII(d2) respectively; LBII derivatives  
chr = chromosomal DNA
Figure 1.7: Plasmid patterns of *Lactobacillus* sp. Y5 and its derivatives after curing trials at sublethal temperatures.

Lane A: Standard; *E. coli* V517  
Lane B: *Lactobacillus* sp. Y5; Parental Strain  
Lane C, D & E: Y5 derivatives (Y5d1, Y5d2, Y5d3)  
chr = chromosomal DNA  
o.f. = open form of plasmid DNA
LITERATURE CITED


Chapter 2

Effect of Nisin on Growth and Survival of 
Listeria monocytogenes in Cottage Cheese and Yogurt*

Noreddine Benkerroum†

and

William E. Sandine

Department of Microbiology
Oregon State University
Corvallis, OR 97331-3804

Running Title: Effect of Nisin on L. monocytogenes

*The part of this chapter dealing with cottage cheese has been published: Inhibitory action of nisin against Listeria monocytogenes, J. Dairy Sci. 71:3237-3245.

†Present address: Department of Food Microbiology, Institute of Agronomy and Veterinary Medicine, Hassan II, Rabat, Morocco.
ABSTRACT

The sensitivity of nine strains of *Listeria* to nisin was determined as well as the minimum inhibitory concentration of nisin necessary to completely inhibit their growth. All strains tested were variably sensitive to nisin and different minimal inhibitory concentration (MIC) values were obtained ranging from 740 to $10^5$ IU/ml in Trypticase Soy Agar (TSA) and from 1.85 to $3.6 \times 10^3$ IU/ml in MRS agar. MIC values obtained on TSA were higher than those obtained on milk agar, MRS agar and Trypticase Soy Broth (TSB). The inhibition of *L. monocytogenes* ATCC 7644 in TSB at different pH values and different nisin concentrations in nonfat dry milk (NDM) (50 IU/ml), in sterilized and non-sterilized cottage cheese ($37 \times 10^2$ and $2.55 \times 10^3$ IU/g, respectively) and in yogurt, also was investigated. This bacterium was completely inhibited in TSB ($37 \times 10^2$ IU/ml) after 24 hours at pH 5 and above and within 24 hours at pH 4.5 and below. Nisin concentrations ranging from 50 to 200 IU/ml inhibited growth of *L. monocytogenes* at pH 6.0 and below but not at pH 6.8. A concentration of 20 IU/ml was inhibitory only at pH 4.5. In NDM, 50 IU/ml was inhibitory to the pathogen at pH 6.8 and below and the activity increased as the pH decreased. In cottage cheese, and in yogurt as well, no *Listeria* survivors were found at 24 hours at $37^\circ$C and $4^\circ$C, and during storage at $4^\circ$C, respectively. Nisin was shown to inhibit yogurt fermentation only at a concentration higher than 50 IU/ml.
INTRODUCTION

Listeria monocytogenes is a food-borne pathogen, which recently has been associated with food-borne disease outbreaks (13, 33). Raw milk has been reported to be a vehicle for this pathogen (16, 22, 23, 27, 31), which can survive the processing of some dairy products (6, 8, 16, 30) or contaminate the plant equipment and thereafter cause post-pasteurization contamination.

Although Bradshaw et al. (3) showed L. monocytogenes would not survive pasteurization, this microorganism has been isolated from pasteurized milk (13, 14), suggesting post-pasteurization contamination or improper pasteurization. However, Doyle et al. (7) showed that this organism survives HTST pasteurization of milk (71.7°C for 15 s). It has also been shown that L. monocytogenes survives the manufacture of cottage (6), Camembert (6, 30) and Cheddar (6, 29) cheese as well as the drying operation to produce NDM (16). Therefore, Listeria may be present in dairy products as a result of post-pasteurization contamination or survival during manufacture; growth of this bacterium can occur under temperature abuse but also under refrigeration conditions. In this regard, Rosenow and Marth (28) showed that L. monocytogenes grows well at 4°C in skim, whole, and chocolate milk and in whipping cream and over a temperature range from 0 to 43°C.

Although many nonspore-forming bacteria are sensitive to nisin, industrial use of this antibiotic is limited to the prevention of spore outgrowth in processed cheeses and canned foods (9, 15). The
earliest use was to prevent the late gas defect in Swiss-type cheeses caused by clostridia (15, 20). It also has been used alone or with subtilin in canned foods to prevent the outgrowth of bacterial spores. Recently, Taylor (40) showed that nisin inhibits the outgrowth of *Clostridium botulinum* spores or impedes toxin production by this microorganism. In addition, Ogden (24) and Ogden and Tubb (25) proposed the use of nisin in brewing to inhibit *Lactobacillus* and *Pediococcus*, the main organisms associated with beer spoilage. In the United States, nisin has been placed on the GRAS list and is approved for use in pasteurized cheese spreads (11). The action of nisin is pH-dependent; it is more effective in low pH systems (5, 20). It is stable at pH 2, where it can resist boiling for 10 min, but it is readily destroyed at pH 11 (18, 20). Campbell and Sniff (4) showed that 200 IU/ml are enough to inhibit *Bacillus coagulans* at pH 5.3 but 560 IU/ml failed to inhibit this bacterium at pH 7.2. According to Henning et al., (17) nisin should be used only in foods where the pH is below 7.0 "to ensure sufficient solubility and stability during processing and storage."

As for the safety of nisin, studies done in USSR, Japan, and England have established its nontoxicity. A joint FAO/WHO committee on food additives stated in 1968 that 3,300,000 IU/kg of body weight does not present any undesirable effects, and consequently, they recognize it as a safe food preservative. Recent public health concerns about *Listeria* in food products and the fact that it is a Gram-positive bacterium prompted us to investigate its sensitivity to nisin.
MATERIALS AND METHODS

Cultures:

Nine strains of Listeria spp were tested for their sensitivity to nisin. The nine strains also were used to determine the minimum inhibitory concentration (MIC) of nisin. They included eight strains isolated from clinical cases and a ninth strain (7644) obtained from the American Type Collection Culture (ATCC), Rockville, MD 20852. The clinical isolates were L. monocytogenes 7644K; L. monocytogenes V7 type 1a; L. monocytogenes 35152; L. monocytogenes Scott A type 4b; L. monocytogenes 1513, L. ivanovii KC1714 type 5; L. ivanovii C194 type b and L. seeligerii LA15.

All strains were grown on slants of Trypticase Soy Agar (TSA) obtained from Baltimore Biological Laboratory (BBL), Baltimore, MD and stored at 4°C. Before each use they were transferred to 10 ml of Trypticase Soy Broth [(TSB) (Bacto-Tryptone, Difco Laboratories, Detroit MI), 15 g; Bacto-soytone (BBL), 5g; NaCl, 5g; and distilled water, 1000 ml] and incubated 16 hours at 37°C.

Nisin was purchased from Aplin and Barrett, Ltd., Trowbridge, England. Its potency was 37 x 10^6 IU/g. The stock solution (37 x 10^3 IU/ml) was prepared by dissolving 0.1 g of nisin in 80 ml of 0.02 N HCl solution and holding at room temperature for 2 hours to complete dissolution. The volume was then made up to 100 ml with 0.02 N HCl and the solution filter sterilized through 0.22-µm Millipore membrane. This solution was stored at -20°C.
Sensitivity Testing:

A modification of the well assay technique described by Fowler et al., (12) was used: melted TSA was tempered to about 46° C and inoculated with 1% overnight culture of *Listeria*. Twenty milliliters then were poured in petri dishes and allowed to harden. Wells (8mm diameter) were then cut into the dishes and filled with 70μl of nisin solution (1mg/ml). These cultures were incubated at 37° C for 24 to 48 hours until inhibition zones were evident. The inhibition zones were then measured with the aid of a graduated ruler.

Determinations of Minimum Inhibitory Concentration:

Determinations of MIC were done on broth and agar media. Agar media used were TSA, MRS (Difco Laboratories, Detroit MI) and milk agar. The agar incorporation method described by Hogg et al. (19) was used. An overnight *Listeria* culture (25μl) was dispensed as a drop on TSA, MRS and milk agar plates containing a given concentration of nisin. Inoculated plates were incubated at 37° C for 24 hours. Nisin-free plates (controls) were inoculated in the same way.

Liquid medium used was TSB. It was dispensed in 10-ml aliquots and nisin was aseptically added to achieve different concentrations and the tubes were inoculated with 0.1 ml (ca. 10⁶ cells/ml) of an overnight *Listeria* culture. A nisin-free tube was used as a control and was inoculated similarly. All tubes were incubated at 37° C. After 24 hours of incubation tubes were checked for turbidity.
Effect of Nisin on *L. monocytogenes* ATCC 7644:

Varying pH with constant nisin concentration: media used were TSB and 10% reconstituted NDM. Three series of test tubes containing 9 ml of TSB each were used. The tubes of each series were adjusted to different pH values (7.0; 6.5; 5.0; 4.5; 4.0 and 3.5) with 85% lactic acid. To the first series (test), 1 ml of nisin stock solution (37 x 10³ IU/ml) and 0.1 ml of 0.6 x 10⁵ CFU/ml *L. monocytogenes* ATCC 7644 overnight cultures were added. The final concentrations of nisin and microorganism in each tube were then 37 x 10² IU/ml (1 mg = 37 x 10³ IU) and 6 x 10² cell/ml. To the second series, only *Listeria* was added to the same concentration, i.e. 6 x 10² cell/ml. The third series was a negative control to test the sterility of the medium; neither nisin nor the microorganism was added. Cultures were incubated at 37°C. *Listeria* count was determined by plate count on TSA at 0, 1, 2, 4, 19 and 24 days.

The same experiment was conducted using reconstituted NDM. However, the final concentration of nisin was 50 IU/ml, the inoculum of *Listeria* was about 10⁶ cells/ml and the pH was adjusted to 6.84, 5.5 and 4.5 with 1 M citric acid. Growth of *L. monocytogenes* was monitored by plate count on TSA at 4, 7, 24 and 48 hours.

Varying pH and nisin concentration: A series of test tubes containing 9 to 10 ml of TSB each were used. The tubes of each series were adjusted to different pH values (6.4, 6, 5.5 and 5) with citric acid. For each pH, a different nisin concentration was used (20, 50, 100, and 200 IU/ml). Tubes were inoculated with 0.1 ml of an overnight
Listeria culture. Negative and positive controls were prepared as described above. Growth of the pathogen was monitored by O.D. determinations at 520 nm at 2, 4, 6, 24 and 48 hours.

Effect of Nisin on *Listeria monocytogenes* ATCC 7644 in Cottage Cheese:

This experiment was done on sterilized and nonsterilized cottage cheese incubated at 37 or 40°C. Two trials were conducted.

The first one was two series of three samples containing 250g of cheese and 50 ml of pasteurized cream (12% milk fat) each. The samples were thoroughly mixed and sterilized for 20 min at 121°C. Nisin and *L. monocytogenes* ATCC 7644 overnight cultures were added to one sample of each series to a final concentration of 2.55 x 10^3 IU/g and 3.5 x 10^5 cell/g, respectively (test). To another sample, the positive control, the culture only was added to the same final concentration. The third sample was a negative control and remained uninoculated and without nisin addition. All these samples were mixed aseptically in a stomacher (Tekmar) for approximately 50 seconds and replaced in sterile 1000-ml flasks. One series was incubated at 40°C, the other at 37°C. They were then sampled for counting *L. monocytogenes* on listeria selective isolation agar (LSI) at 0, 1, 2, 5, 9, 14, 24 and 30 days. LSI (37) consisted of: TSA (Difco), 45g; yeast extract (Difco), 5 g; bromocresol purple (Difco) 0.04 g; and esculin (Sigma Chemical Co., St. Louis, MO), 5 g; supplemented with filter sterilized acriflavine hydrochloride (Sigma) and nalidixic acid (Sigma). The latter two ingredients were in solution in 0.1 N NaOH to
final concentrations of 0.010 and 0.040 mg/ml, respectively. Filter-sterilized aqueous solution of polymixin B sulfate (Sigma) to a final concentration of 16 IU/ml was also added. When no *Listeria* was found in a 1-ml sample by the plate count method, we used the FDA method for *Listeria* isolation described by Lovett et al. (22). A 25-ml sample was enriched in 225 ml of a selective enrichment medium described by Bannerma and Billie (2) and incubated at 30°C. At 1 day and 7 days, a 10-µl loop from the enriched culture was streaked on LSI agar and incubated for 24 to 48 hours.

In the second trial, three samples were also prepared (test, positive control, and negative control) in the same way as in the first case except that they were not sterilized. Nisin concentration in the test and the initial concentration of *Listeria* in the test and the positive control were also the same. The nonsterilized cottage cheese was incubated at 4°C and sampled for plate count on LSI agar at 0, 1, 2, 5, 9, 14, 24, and 30 days.

**Effect of Nisin on Yogurt Fermentation:**

As *Lactobacillus bulgaricus* used in yogurt starter cultures was reported to be sensitive to nisin (24, 25), this preliminary experiment was carried out to determine the concentration of nisin which can be used in yogurt without affecting its normal processing, especially acid production.
Yogurt trials were made as follows: Whole dry milk was reconstituted at 17% and pasteurized in water bath at 80°C (internal temperature) for 30 min. Of the pasteurized milk, 100 milliliters were dispensed in 120-ml waxed paper cups and inoculated with 2% activated commercial yogurt starter consisting of *Lb. bulgaricus* and *Streptococcus thermophilus* (1:1) (Redi-set, Hansen Laboratories, Inc. Milwaukee, WI). The starter culture was activated in sterile NDM according to the manufacturer's recommendations. Nisin was added to the cups to a final concentration of 10, 20, 40, 50 and 100 IU/ml. A sample not containing nisin was used as a control. The containers were incubated at 43°C until coagulation, (6 to 7 hrs), then transferred to the refrigerator for the rest of the experiment. The pH and acidity were measured every hour until coagulation then at 1, 13, and 15 days. pH measurements were done with a Crison pH meter using an ingold combination electrode. The acidity was measured by titration with N/10 NaOH solution in presence of 1% phenolphthalein solution.

**Effect of Nisin on Listeria monocytogenes in Yogurt:**

Two yogurt cups were prepared as described above. Nisin was added to one of them to a final concentration of 50 IU/ml. The second was a positive control. A third cup containing only pasteurized reconstituted NDM was also used as a second positive control. All cups were inoculated with a 0.1 ml (c.a. 10⁵ IU/ml) of an overnight *Listeria* culture. Samples were incubated at 43°C until those containing the starter coagulated. They were then held at 4°C
and sampled for plate count on ASLM (1) at 1, 2, 3, 13 and 15 days. When no *L. monocytogenes* was found in a 1-ml sample, enrichment procedure was followed as described above.
RESULTS AND DISCUSSION

Susceptibility Testing:

The well diffusion assay for susceptibility testing showed that *Listeria* is inhibited by nisin. The mean of inhibition zone diameters for each strain tested is shown in Table 2.1. All strains were inhibited by nisin, but important strain differences were observed in the degree of inhibition showing that some *Listeria* strains are more sensitive to nisin than others: *L. monocytogenes* ATCC 7644 and *L. ivanovii* KC 1714 type 5 were the most sensitive (18 mm diameter) while, *L. monocytogenes* V7 type 1a was most resistant (10mm diameter).

Determination of the Minimum Inhibitory Concentration:

The MIC ranges of the nine strains tested on different media are shown in Table 2.1 These results show again that *L. monocytogenes* is the most vulnerable strain to nisin among those tested. It has the lowest MIC (740 IU/ml on TSA), followed by *L. ivanovii* KC 1714 type 5. The *L. monocytogenes* V7 type 1a strain had the highest MIC (1.18 x 10^5 IU/ml on TSA). In general, the MIC ranges obtained were relatively high and subject to variations, depending on the assay conditions, mainly pH and composition of the medium. The pH of TSA used in this experiment was 7.3, which is not optimum for nisin action as stated earlier. Also, MIC ranges obtained on MRS lactobacilli agar were much lower than those obtained on TSA (Table 2.2). One explanation for this is the lower pH of MRS (6.8), which is
more suitable for nisin action than pH 7.3. Also, MRS contains some components which may synergistically inhibit *Listeria* with nisin increasing the overall effect. Sodium acetate contained in this medium is indeed inhibitory to a number of gram-positive and gram-negative bacteria. Table 2.2 shows also that *L. monocytogenes* ATCC 7466 had lower MIC on milk agar and in TSB than on TSA. The lower MIC in milk agar may be also due to a synergistic action of a milk inhibitor system (lactenins) and nisin. As for the difference between TSB and TSA, an explanation would be that nisin has a better access to the bacterium in the liquid than in the solid state of the medium. These results suggest that one must be careful when wanting to minimize nisin addition to products based on MIC determinations under specific conditions. Dairy products where nisin has been mostly used as a preservative or to extend the shelf-life (9, 20) seem to be suitable for nisin action and 10 to 100 IU/ml are generally used in practice. As regards regulations concerning the use of nisin as a food preservative, most countries where it is permitted allow either 12.5 mg/kg (about 5000 IU/g) or do not limit the amount (10, 20).

Effect of Nisin on *Listeria monocytogenes* ATCC 7466 at Different pH Values

Growth of *L. monocytogenes* at different pH values in absence or presence of $37 \times 10^2$ IU/ml of nisin is shown in Figure 2.1. *L. monocytogenes* grew well without nisin at pH of 5.5 to 7.0. At pH 5.0, growth was slow but not completely inhibited. After 24 hours,
no *Listeria* were found in a 1-ml sample; however, regrowth (6.3 x 10² cell/ml) was observed by the 19th day at this pH. The inhibitory action of pH became clear at pH 4.5 and below. The pathogen was eliminated from the sample after 48 hours at pH 4.5 and after only 24 hours at pH 3.5. No regrowth was observed at pH lower than 5.0.

Similar results were reported by Conner et al., (5) and Sorrells et al., (38), who showed that *L. monocytogenes* is completely inhibited at pH 4.6 and below when lactic acid is used to adjust the pH.

Figure 2.1 shows also the growth of *L. monocytogenes* ATCC 7466 in TSB with 37 x 10² IU/ml of nisin. No survivors were found after 24 hours in a 1 ml sample at all pH values. At pH 7 and pH 6.5, an increase of *Listeria* count was observed in the first 24 hours to about 10⁴ CFU/ml and then no *Listeria* were detected in 1 ml within the next 24 hours.

Similar results were obtained with reconstituted NDM (Figure 2.2, and Table 2.3). From Figure 2.2 it may be seen that counts of *L. monocytogenes* kept increasing at pH 6.8 in the controls (without nisin), while they decreased steadily in test samples containing 50 IU/ml of nisin. At pH 4.5, a decrease in *Listeria* counts was observed in both test and control samples; however, the pathogen was eliminated from the test sample within 24 hours while in the control, few cells were still viable even at 48 hours. The same behavior was observed at pH 5.5 and 5.0 (Table 2.3). These results suggest that 50 IU/ml of nisin can be effective in the control of *L. monocytogenes* in milk and dairy products.
In view of these results taking into account that, in practice, high concentrations of nisin may not always be possible to use, this experiment was carried out using different combinations of pH and nisin concentrations. Results are shown in Figure 2.3. At pH 6.8, no concentration was inhibitory to *L. monocytogenes*; no significant difference (P <0.01) between O.D. reached after 48 hours in samples containing up to 200 IU/ml and the control, was observed. At pH 6 and 5.5, however, 50, 100 and 200 IU/ml were all effective. A concentration of 20 IU/ml had no significant (P < 0.01) effect on *L. monocytogenes* at pH 6.8, and 5.5. This concentration however was inhibitory at pH 5. These data show that much less nisin is needed to control *L. monocytogenes* in low pH than in high pH systems, either because nisin is more effective at low pH, as has been shown earlier (4, 17), or because of an additive effect of acidity and nisin action. However, in contrast to the report of Henning et al., (17), nisin was still active at pH 7.0 (Figure 2.1), at least, at high concentration. According to these authors, nisin is less soluble at pH 7.0, which impeded its effectiveness. Wei and Hansen (41) also investigated the effect of pH on nisin solubility and found that it decreases exponentially from pH 2 to 6 and that it is almost insoluble around pH 8. Hurst (20), however, reported that a high protein concentration enhances the solubilization of nisin. According to Eapen et al., (9) the solubility of nisin in water at pH 7.0 is 75 µg/ml (the equivalent of 2.8 x 10³ IU/ml), which is sufficient to inhibit all microorganisms susceptible to this substance. In food preservation, in general, 10 to 500 IU/g has been recommended (9). Moreover, it
has been shown that an average of 2 to 10 IU/ml was enough to inhibit a cell concentration of $2 \times 10^5$/ml (18). Some microorganisms, however, need a high nisin concentration to be inhibited. In this regard, Taylor (40) showed that 50 to 200 μg/g (1.9 x 10^3 to 7.6 x 10^3 IU/g) were necessary to inhibit *C. botulinum* in various canned foods. Also, Mohamed et al., (23) showed that only 32 IU/ml were necessary to inhibit *L. monocytogenes* 4379 at pH 7.4 and 37°C. They also showed that the sensitivity of this strain decreases when the temperature of incubation decreases: 256 IU/ml are required to inhibit completely *L. monocytogenes* 4379 growth at 22°C and pH 7.4. However, this amount is 16-fold reduced at pH 5.5 at the same temperature. In fact, solubility of nisin in dairy products does not seem to be a concern, because most have a low pH and they contain enough proteins to help solubilize nisin. Furthermore, nisin has already been used successfully in cheeses, as mentioned earlier. In other food systems, however, protein-nisin interactions may occur to reduce the effectiveness of nisin.

**Effect of Nisin on *Listeria monocytogenes* ATCC 7644 in Sterilized and Nonsterilized Cottage Cheese.**

Figure 2.4 shows the growth curves of the pathogen in the positive control and the test samples of the sterilized cottage cheese at 4 and 37°C. No *Listeria* could be found in 1 ml of sample after 1 day at 4°C as well as at 37°C. Furthermore, the pathogen could not be recovered by the FDA method for *Listeria* isolation either after 1 day or after 1 week, which confirms that it was killed by nisin, not only inhibited
or injured. The same figure shows also that *Listeria* grows well in sterilized cottage cheese (positive sample) despite its low pH, which was in our case 4.9 to 5.0, while the synthetic medium showed that the pathogen was greatly, although not completely, inhibited (regrowth was observed by the 19th day) (Figure 2.1) at pH 5. This may be explained by the richness of cottage cheese that provides *Listeria* with some growth factors not found in the synthetic medium, or by the protective effect that cheese proteins may have for the pathogen as it has been shown with *Clostridium botulinum*, which can grow in canned foods at lower pH than in synthetic media. It may be a combination of these two factors as well.

Table 2.4 summarizes the effect of nisin on *Listeria* in nonsterilized cottage cheese at 4°C. It appears that the cheese starter bacteria are not inhibited by LSI medium. However, the latter form larger colonies and ferment the esculin more slowly when used as a fermentable carbohydrate in the medium. Some suspicious colonies (smaller and turn yellow faster) were selected and Gram stained. Such colonies were Gram-positive cocci and therefore assumed to be starter bacteria. The pathogen was not frequently found in the positive control either; one or two colonies, at the most out of 10 were *Listeria*, sometimes none were *Listeria*. This suggests that starter bacteria exhibit an antagonistic effect against *Listeria*, which is no surprise; in fact, the inhibitory effect of lactic acid bacteria on pathogens and spoilage microorganisms is now well known. Nonetheless, the inhibition of the pathogen by starter bacteria is not a guarantee of the safety of this cheese. Some cells can survive as it
was found here and elsewhere (5, 31, 37). However, the addition of nisin not only inhibits the growth of *Listeria*, but also kills the bacterium. The antibiotic is, therefore, able to protect this product from *Listeria* when contaminated either from the raw milk or as a result of post-pasteurization contamination. Most important, nisin conserves its effectiveness at a low temperature for a long time (15), ensuring protection of the product against *Listeria* contamination when it is stored under refrigeration. Furthermore, nisin delays the growth of other spoilage psychrotrophs that can exist or contaminate cottage cheese. In effect, in this experiment, nonsterilized cottage cheese samples without nisin spoiled 1 wk earlier than those containing the antibiotic (the spoilage was judged by the alteration of the physical appearance and smell). Bacterial counts were also lower in the test than in control samples (Table 2.4).

**Effect of Nisin on Yogurt Fermentation:**

Figure 2.5 shows the pH decrease and the increase of acidity during yogurt fermentation and with different concentrations of nisin compared to a positive control (without nisin). It may be seen from this figure that nisin concentration of 50 IU/ml and below had no noticeable effect on yogurt fermentation. The pH dropped and the acidity increased in the same way as in the control. Furthermore, milk coagulation in all these samples was normal; it occurred from 6 to 7 hours and the curd was firm and without syneresis. However, in the samples containing 100 IU/ml of nisin, fermentation was greatly retarded and the curd had an abnormal, viscous body. After 15
days, the acidity and the pH of these samples were, respectively, only 0.60% and 4.5 on the average, while in the control it was 1%. This is due to the fact that nisin is inhibitory to *Lb. bulgaricus*; therefore, addition of this substance to yogurt during fermentation to a certain level would lead to an unbalanced rod-coccus ratio. These results suggest that if nisin is to be used for yogurt preservation, the amount added should be 50 IU/ml or less.

**Effect of nisin on *L. monocytogenes* in yogurt:**

Figure 2.6 shows the behavior of *L. monocytogenes* in yogurt in the presence and absence of nisin during storage at 4°C. Although a significant decrease in *Listeria* counts was observed in yogurt without nisin, the pathogen survived manufacture and 15 days of storage at 4°C. However, in yogurt containing 50 IU/ml of nisin, no *Listeria* was found in a 1-ml sample at 24 hours and thereafter. Also, no survivors were resuscitated by the enrichment procedure. Survival of pathogens in fermented dairy products, in spite of the antagonistic effect of lactic acid bacteria used as starter cultures, is well documented (6, 29, 30, 31, 34, 35). In yogurt and in other dairy products fermented with the same starter [e.g. *Lb. bulgaricus*, and *Streptococcus thermophilus* (ST:LB::1:1)] such as some cultured milks and Feta cheese, *L. monocytogenes* was shown to survive manufacture and storage (26, 34, 35). Papageorgiou and Marth (26) showed that *L. monocytogenes* could survive the manufacture and more than 90 days of storage at 4°C in Feta cheese. This bacterium was also shown to survive in cultured milk fermented with STLB and
in yogurt from 1 to 12 weeks and 1 to 12 days, respectively (34). The same authors showed that survival of *L. monocytogenes* in yogurt depends on the size of both *Listeria* and starter culture inocula, the final pH reached, the temperature and duration of the fermentation, and *Listeria* strain. Shaack and Marth (34) showed that *L. monocytogenes* survives only between 9 to 15 hours during the actual fermentation process of yogurt. However, in a typical yogurt fermentation, which lasts 4 to 6 hours, they showed that this bacterium was able to grow during fermentation and then survive during storage at 4°C (34). Lammerding and Doyle (21) also could recover *L. monocytogenes* from yogurt after 7 days of storage at 4°C, although the initial inoculum was relatively low (about 32 x 10^2 CFU/ml). Survival of *L. monocytogenes* in yogurt in spite of the low pH and the antagonistic activity of the starter culture, may be explained by the protective effect of the milk casein on the pathogen, as was shown with *Salmonella typhimurium* (32). These data and those reported elsewhere (6, 10, 26, 35) show that food processors should not rely on pasteurization and fermentation only to insure full protection and safety of fermented milk products. Some food additives may also be used in addition along with Good Manufacture Practices. In our case, nisin proved efficient in controlling growth of *L. monocytogenes* in both cottage cheese and yogurt. In the latter case, however, the amount of nisin to be used is crucial and should be sufficient to inhibit the pathogen without harming *Lb. bulgaricus*; otherwise, the product will be defective.
This work shows that in addition to the classical use of nisin to prevent outgrowth of bacterial spores in foods, it can also be used to prevent growth of some food-borne pathogens of major concern such as \textit{L. monocytogenes}. Growth of this bacterium that can occur at a relatively low pH and under refrigeration can be overcome by nisin addition. The sensitivity of \textit{Listeria} to nisin was shown to be somewhat strain dependent, which should be taken into account in determining amounts to be added in order to offer a safety factor. Also, since nisin has no known toxicity for humans, its use as a food additive in the United States to inhibit pathogens warrants further consideration.
Table 2.1: Diameter of inhibition zones appearing on Petri plates seeded with *Listeria* sp. as caused by nisin (70 µl added to assay well).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Inhibition Zone Diameter&lt;sup&gt;1&lt;/sup&gt; (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. monocytogenes</em> 7644K</td>
<td>12 (.71)&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>L. monocytogenes</em> 35152</td>
<td>11 (.41)</td>
</tr>
<tr>
<td><em>L. monocytogenes</em> V7 type 1a</td>
<td>10 (.90)</td>
</tr>
<tr>
<td><em>L. monocytogenes</em> Scott A type 4 b</td>
<td>11 (.73)</td>
</tr>
<tr>
<td><em>L. ivanovii</em> C194 type b</td>
<td>11 (.16)</td>
</tr>
<tr>
<td><em>L. monocytogenes</em> 15313</td>
<td>11 (.16)</td>
</tr>
<tr>
<td><em>L. seeligerii</em> LA 15</td>
<td>12 (1.08)</td>
</tr>
<tr>
<td><em>L. ivanovii</em> KC1714 type 5</td>
<td>18 (1.41)</td>
</tr>
<tr>
<td><em>L. monocytogenes</em> ATCC 7644</td>
<td>18 (1.47)</td>
</tr>
</tbody>
</table>

<sup>1</sup>Mean values of four determinations

<sup>2</sup>Standard deviation values in parenthesis
Table 2.2: Minimum inhibitory concentration (MIC) (pH 7.3) of nisin on *Listeria monocytogenes* ATCC 7644 determined on Trypticase Soy Agar (TSA), MRS agar at pH 6.8, milk agar and Trypticase Soy Broth (TSB).

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TSA</td>
</tr>
<tr>
<td><em>L. monocytogenes</em> 7644K</td>
<td>$1.48 \times 10^4$</td>
</tr>
<tr>
<td><em>L. monocytogenes</em> 35152</td>
<td>$1.48 \times 10^4$</td>
</tr>
<tr>
<td><em>L. monocytogenes</em> V7 type 1a</td>
<td>$1.18 \times 10^5$</td>
</tr>
<tr>
<td><em>L. monocytogenes</em> Scott A type 4 b</td>
<td>$1.18 \times 10^4$</td>
</tr>
<tr>
<td><em>L. ivanovii</em> C194 type b</td>
<td>$74 \times 10^3$</td>
</tr>
<tr>
<td><em>L. monocytogenes</em> 15313</td>
<td>$1.48 \times 10^4$</td>
</tr>
<tr>
<td><em>L. seeligerii</em> LA 15</td>
<td>$1.48 \times 10^4$</td>
</tr>
<tr>
<td><em>L. ivanovii</em> KC1714 type 5</td>
<td>$14.8 \times 10^2$</td>
</tr>
<tr>
<td><em>L. monocytogenes</em> ATCC 7644</td>
<td>740</td>
</tr>
</tbody>
</table>
Table 2.3: Effect of pH and added nisin (50 IU/ml) on growth of *Listeria monocytogenes* (log CFU/ml)\(^1\) in reconstituted nonfat dry milk incubated at 37°C.

<table>
<thead>
<tr>
<th>Time(h)</th>
<th>pH</th>
<th>+nisin</th>
<th>-nisin</th>
<th>+nisin</th>
<th>-nisin</th>
<th>+nisin</th>
<th>-nisin</th>
<th>+nisin</th>
<th>-nisin</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
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<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td>4</td>
<td>6.7(0.29)(^2)</td>
<td>7.5(0.53)</td>
<td>6.4(0.59)</td>
<td>6.7(0.56)</td>
<td>5.6(0.82)</td>
<td>6.2(0.16)</td>
<td>4.7(0.37)</td>
<td>5.2(0.70)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>7.8(0.18)</td>
<td>8.9(0.29)</td>
<td>6.6(0.66)</td>
<td>7.1(0.26)</td>
<td>5.7(0.26)</td>
<td>5.8(0.48)</td>
<td>4.5(0.38)</td>
<td>4.7(0.29)</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>3.8(0.63)</td>
<td>9.5(0.41)</td>
<td>2.8(0.55)</td>
<td>6.9(0.42)</td>
<td>3.3(0.53)</td>
<td>5.8(0.77)</td>
<td>0.0(0)</td>
<td>2.8(0.73)</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>1.5(0.50)</td>
<td>9.5(0.29)</td>
<td>1.2(0.30)</td>
<td>8.3(0.33)</td>
<td>0.0(0)</td>
<td>2.4(0.43)</td>
<td>0.0(0)</td>
<td>0.8(0.85)</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)Values are means of four determinations.

\(^2\)Standard deviations in parenthesis.
Table 2.4: Influence of nisin on microbial counts developing on Listeria selective isolation medium in non-sterilized cottage cheese contaminated with *L. monocytogenes* ATCC 7644 during incubation at 40°C.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>5</th>
<th>9</th>
<th>14</th>
<th>24</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nisin added</td>
<td>.04</td>
<td>.08</td>
<td>.3</td>
<td>.2</td>
<td>4.0</td>
<td>.2</td>
<td>.1</td>
<td>.1</td>
</tr>
<tr>
<td>No nisin</td>
<td>.04</td>
<td>.1</td>
<td>.8</td>
<td>.6</td>
<td>3.0</td>
<td>3.0</td>
<td>2.0</td>
<td>3</td>
</tr>
<tr>
<td>Control</td>
<td>---</td>
<td>.09</td>
<td>.9</td>
<td>.5</td>
<td>.06</td>
<td>.02</td>
<td>4.0</td>
<td>3</td>
</tr>
</tbody>
</table>

15 x 10⁵ cell/g of *L. monocytogenes* added.

2While starter bacteria present in the cottage cheese grew on LSI medium, *L. monocytogenes* could not be found on plates of samples from the cheese containing nisin.

3Colonies were cheese starter bacteria and *L. monocytogenes*.

4No *L. monocytogenes* added, so colonies were only cheese starter bacteria.
Figure 2.1: Effect of pH and added nisin (37 x 10² IU/ml) on growth of *Listeria monocytogenes* in Trypticase Soy Broth incubated at 37°C.
Figure 2.2: Effect of pH and added nisin (50 IU/ml) on growth of *Listeria monocytogenes* ATCC 7644 (log CFU/ml) in reconstituted non fat dry milk (NDM) incubated at 37°C.
Figure 2.3: Combined effect of pH and nisin concentration on growth of *Listeria monocytogenes* in Trypticase Soy Broth incubated at 37°C
Figure 2.4: Growth of *Listeria monocytogenes* 7644 at 4 and 37°C in the presence and absence of nisin (2.55 x 10³ IU/g) in sterilized cottage cheese.
Figure 2.5: Effect of nisin on yogurt fermentation.

A: Control (no nisin added)
B: 20 IU/ml of added nisin
C: 50 IU/ml of added nisin
D: 100 IU/ml of added nisin
Figure 2.6: Effect of nisin (50 IU/ml) on *Listeria monocytogenes* in yogurt.

LM = *Listeria monocytogenes*
SC = Starter Culture
N = Nisin


Chapter 3

Effect of Nisin on the Keeping Quality of Feta Cheese

Noreddine Benkerroum*

and

William E. Sandine

Department of Microbiology
Oregon State University
Corvallis, OR 97331-3804

Running Title: Nisin and Feta Cheese

*Present address: Department of Food Microbiology, Institute of Agronomy and Veterinary Medicine, Hassan II, Rabat, Morocco.
Nisin was added to Feta cheese during cheesemaking fermentation to achieve a final concentration of 40 IU/ml in an attempt to extend cheese shelf-life. At this concentration, nisin had no adverse effect on the cheese starter bacteria. Neither acid production during fermentation, nor chemical composition of the final Feta cheese was affected. No significant effect (P <0.01) was observed on salt, fat or ash contents. Although the total dry matter was significantly reduced, its value along with the fat in the dry matter (FDM) still met Greek regulatory standards. Shelf-life, however, was reduced to less than 47 days due to an intensive growth of Gram-negative psychrotrophic bacteria which were not inhibited by nisin.
INTRODUCTION

Feta is a soft, white cheese. It originally was a Greek, home-made cheese characterized by "its smooth, creamy, soluble and sliceable body and pleasant acidic, salty and mild rancid flavor" when ripened (9). For a long time, manufacture of Feta cheese relied on the natural flora of milk during the fermentation; therefore, its composition and flavor were not consistent and varied greatly among localities, climatic conditions and traditions. Furthermore, defects in body and flavor were common, as one would expect. Feta cheese is normally made from sheep or goat milk or blends of both. However, production of these two kinds of milk is not sufficient to yield significant quantities of cheese. Therefore, cow milk now is used as well. Similar dairy products have been described in Near Eastern countries (10). In Egypt, Domiati cheese is very similar to Feta cheese, the main difference being in the salting procedure. Feta cheese is salted in 12% brine, while for Domiati cheese, salt is directly added to the milk before fermentation to a final concentration of 5 to 15% (10). In 1964, Etthymiou and Mattick (9) developed a procedure to manufacture Feta cheese from pasteurized cow milk using selected starter cultures. The best results were obtained with a mixed starter culture composed of lactococci and lactobacilli. Yogurt starter (e.g. Lactobacillus bulgaricus and Streptococcus thermophilus) has also been used successfully (19).

The shelf-life of Feta cheese is variable, depending on raw milk quality and the degree of cleanliness during manufacture.
Papageorgiou and Marth (19) showed that high quality Feta cheese could be conserved for more than 90 days. In some cases the shelf-life of Feta cheese may be less, especially if the curd has been cut before sufficient acid has been produced (9). It is well known that at least 25% of the world's food supply spoils before it can be consumed. The cost of this, while difficult to accurately estimate, is nonetheless astronomical, especially in lost income (5). Therefore, intensive research work has been done to extend the shelf-life of perishable foods. The most common means used are heat treatments, refrigeration, use of food additives, bacteriocins or bacteriocin producer microorganisms and the use of viable lactic acid bacteria. Nisin has long been used for this purpose in dairy products, meats, canned foods, and beverages (4, 6, 8).

In the present study an attempt was made to extend the shelf-life of Feta cheese made from pasteurized cow milk by addition of nisin.
MATERIALS AND METHODS

Starter Culture:

Commercial lyophilized starter culture (Visbyvac, yogurt 231) consisting of *Lb. bulgaricus* and *Str. thermophilus* (1:1) was obtained from Laboratorium Wiesby (Germany). The starter was regenerated in sterile reconstituted NDM, which was incubated overnight at 37°C.

Nisin:

Nisin was purchased from Applin and Barett, Trowbridge, England. The stock solution was prepared as described earlier (Chapter 1 of this thesis).

Manufacture of Feta Cheese:

Feta cheese was manufactured as described by Papageorgiou and Marth (19). Two batches were simultaneously made in each of three trials. In each batch, whole cow milk (10l) was pasteurized (75°C for 16 seconds), tempered and placed in stainless steel vats partially submerged in hot water (35-40 °C) to maintain the milk temperature at 35°C. Starter culture was then added (1% v/v) along with 0.01% (w/v) of calcium chloride. When the pH was reduced to 6.4, 2.5 ml of 1/10,000 strength calf rennet was added. To one batch, nisin was added at the same time as rennet to a final concentration of 40 IU/ml, followed by thorough mixing. No nisin was added to the
second batch which served as a control. The coagulum was cut with 0.63-cm knives 40 to 50 min after rennet addition. Curds were then transferred into rectangular perforated stainless steel forms (30 x 22 x 12 cm) and allowed to drain at room temperature (c.a. 22°C) for 6 hours. Forms were turned twice at 2-hour intervals. After draining, cheese was cut into six pieces (10 x 11 x 12 cm) and placed into 12% salt brine for 24 hours at room temperature. The pieces of cheese were then cut in half (10 x 5.5 x 12 cm) and placed in 6% salt brine. Cheese was ripened in this brine at room temperature for 4 days. After ripening, each piece was placed into a sterile metallic can and covered with 6% brine and stored at 4°C.

**Chemical Analysis of Feta Cheese:**

Measurement of pH during fermentation and storage of cheese by using a Corning pH meter equipped with a combination electrode. Total dry matter (TDM), fat, salt and ash contents were determined as described in Standard Methods for the Examination of Dairy Products (22).

**Microbiological Analysis of Cheese:**

Samples of milk, curd and cheese were serially diluted in a sterile 2% sodium citrate solution and plate counts were performed on appropriate agar media. In the case of curd and cheese, to achieve the first dilution (1:10), 10 grams were weighed and placed into a sterile stomacher bag containing 90 ml of sterile 2% sodium citrate
solution, then blended in a Stomacher for 2 to 4 min. Groups of microorganisms enumerated in this study were: total aerobes, coliforms, psychrotrophs and lactic acid bacteria (LAB). Media and incubation conditions used are summarized in Table 3.1.
RESULTS

Effect of Nisin on the Manufacture of Feta Cheese:

Table 3.2 shows variations in the pH occurring during manufacture and storage of Feta cheese with and without nisin added. The pH varied similarly both in control (nisin free) and test (plus 40 IU/ml of nisin) samples. It decreased to pH 4.55 and 4.70 at 6 days, then increased to 4.73 and 5.01, respectively, at 47 days.

Effect of Nisin on the Chemical Composition of Feta Cheese:

Table 3.3 summarizes results on determinations of Fat in the Dry Matter (FDM), salt, ash and Total Dry Matter (TDM) content in cheese after brining in 6% NaCl (e.g. the first day of storage). These data show that addition of 40 IU/ml of nisin during Feta cheese manufacture had no significant effect (P < 0.01) on the fat, NaCl or ash contents. However, TDM was significantly (P < 0.01) lower in test samples than in control. The average TDM in the control and in the test samples were 44.95 (± 0.83) and 43.42% (± 0.28), respectively.

Effect of Nisin on the Shelf-Life of Feta Cheese:

During manufacture and storage of Feta cheese, microbiological analysis consisting of enumerations of total count, coliforms, psychrotrophs and LAB (Figure 3.1: a, b, c, and d) were performed. Cheese appearance was assessed on a regular basis during storage at
$4^{\circ}C$. Figure 3.1 shows that all groups of microorganisms enumerated in this study grew well in Feta cheese, regardless of whether or not it contained nisin. This figure also shows that coliforms and psychrotrophs were not found in the pasteurized milk but were detected in the cheese in relatively high numbers, higher in the test than in the control (Figure 3.1, b, and c). The average coliform counts at 47 days were $9.8 \times 10^6$ CFU/ml in the test and $1.8 \times 10^5$ CFU/ml in control. The average psychrotroph counts in the test and in the control were $1.8 \times 10^6$ CFU/ml and $6.8 \times 10^4$ CFU/ml, respectively. Total and LAB counts varied in similar ways. They were maximum within 5 days and remained relatively constant during storage (Figure 3.1: a and d).

The appearance of cheese containing nisin was altered after 40 days of storage. The surface of the cheese became slimy and soft. This alteration is typically caused by *Pseudomonas* bacteria.
DISCUSSION

Results of this study showed that addition of 40 IU/ml of nisin to Feta cheese had no adverse effect on its manufacture. Decrease of the pH normally observed during lactic fermentation was not affected. The increase of the pH after 6 days (Table 3.3) was observed both in control and test batches; therefore, it could not be attributed to a nisin effect. Similar findings were reported by Ethymiou and Mattick (9), who showed that the pH of Feta cheese increased from 4.7 to 5.1, 5.3 during one month of storage. Papageorgiou and Marth (19), however, showed that the pH of Feta cheese was about 4.3 after brining and this value was maintained through the whole period of storage (90 days). The increase of the pH in Feta cheese after 6 days may be due to contamination of the cheese by microorganisms able to deaminate amino acids, resulting in release of ammonia. Such microorganisms are widely distributed in nature (14). In this regard *Pseudomonas* was reported to produce ammonia from arginine (23, 28). Also, the chemical composition of Feta cheese was not affected by nisin addition, except for the TDM, which was lower in the test than in the control. However in both cases FDM met Greek regulatory standards which provide that it cannot be lower than 43%. The average value of salt content in the control and test cheeses (3.1% ± 0.89 and 3.01 ± 0.74, respectively) were higher than the normal salt content in commercial Feta cheese, which is about 2.5% (18). However, there is no standard for the amount of salt. Greek regulations describe only the salting
procedure; therefore great variations in salt content of Feta cheese should be expected and are, in fact, tolerated.

As for shelf-life, findings of this study showed that 40 IU/ml of nisin did not extend the shelf-life; on the contrary, it was reduced. Numbers of coliforms and psychrotrophs were higher in cheese containing nisin than in cheese without nisin (Figure 1, b and c). It is well known that nisin inhibits only Gram-positive bacteria and has no effect on Gram-negative bacteria, including coliforms and *Pseudomonas* (6, 8, 15, 25). Nisin may encourage growth of these microorganisms by limiting the inhibitory action of the lactic starter bacteria against them. *Lb. bulgaricus* has been shown to produce inhibitory substances against a variety of microorganisms including *Pseudomonas* (1, 3), which appear to be responsible for the altered cheese quality. According to Taylor and Somers (27), the use of lactobacilli is more efficient to preserve bacon than nisin. Although nisin has been shown to extend the shelf-life of many foods, including beverages (6, 8, 17, 18), dairy products (2, 6, 7, 8, 12, 16, 24), canned foods (4, 7, 13, 26) and meat (6, 8, 21, 27), in other cases it has been shown to have limited success (8, 20, 27) or even an adverse effect, especially in fermented products where the starter cultures are sensitive to nisin. In this regard we showed earlier (Chap II, this thesis) that a relatively high concentration of nisin (more than 50 IU/ml) impedes the yogurt fermentation. Also, in order for nisin to extend efficiently the shelf-life of food products, it is necessary to maintain Good Manufacturing Practices because this bacteriocin will not inhibit the growth of many spoilage bacteria,
particularly *Pseudomonas*, which are common causes of dairy product deterioration (5).
Table 3.1: Media and incubation conditions used in the enumeration of different groups of microorganisms in Feta cheese.

<table>
<thead>
<tr>
<th>Group of Microorganisms</th>
<th>Medium</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total count</td>
<td>Plate Count Agar (Difco)</td>
<td>37°C for 72 hours</td>
</tr>
<tr>
<td>Coliforms</td>
<td>VRBA (Difco)(^1)+1,5% agar (Difco)</td>
<td>32°C for 24 hours</td>
</tr>
<tr>
<td>Psychrotrophs</td>
<td>Plate Count Agar (Difco)</td>
<td>7°C for 10 days</td>
</tr>
<tr>
<td>LAB(^2)</td>
<td>MRS (Difco)+1,5% agar (Difco)</td>
<td>30°C for 24 hours</td>
</tr>
</tbody>
</table>

\(^1\)VRBA = Violet Red Bile Agar  
\(^2\)LAB = Lactic Acid Bacteria
### Table 3.2: Variation of pH during manufacture and storage of Feta cheese with and without nisin added.

<table>
<thead>
<tr>
<th>Time</th>
<th>Control (without nisin)</th>
<th>Test (+40 IU/ml of nisin)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
<td>SD¹</td>
</tr>
<tr>
<td>0</td>
<td>6.65</td>
<td>0.15</td>
</tr>
<tr>
<td>0.5</td>
<td>6.56</td>
<td>0.11</td>
</tr>
<tr>
<td>0.7</td>
<td>6.45</td>
<td>0.04</td>
</tr>
<tr>
<td>1²</td>
<td>6.41</td>
<td>0.02</td>
</tr>
<tr>
<td>1.70³</td>
<td>5.46</td>
<td>0.13</td>
</tr>
<tr>
<td>5.5</td>
<td>5.19</td>
<td>0.08</td>
</tr>
<tr>
<td>7.5</td>
<td>5.24</td>
<td>0.24</td>
</tr>
<tr>
<td>24</td>
<td>4.74</td>
<td>0.21</td>
</tr>
<tr>
<td>5 (days)</td>
<td>4.65</td>
<td>0.16</td>
</tr>
<tr>
<td>6 (days)</td>
<td>4.55</td>
<td>0.05</td>
</tr>
<tr>
<td>11 (days)</td>
<td>4.68</td>
<td>0.17</td>
</tr>
<tr>
<td>47 (days)</td>
<td>4.73</td>
<td>0.1</td>
</tr>
</tbody>
</table>

¹Standard deviation (±)
²Rennet addition
³Curd cutting
Table 3.3: Chemical analysis of Feta cheese\textsuperscript{1} with and without nisin added.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Control (nisin, free)</th>
<th>Test (+40 IU/ml of nisin)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
<td>SD\textsuperscript{2}</td>
</tr>
<tr>
<td>TDM (%)\textsuperscript{3}</td>
<td>44.95</td>
<td>.83</td>
</tr>
<tr>
<td>FDM (%)\textsuperscript{4}</td>
<td>47.07</td>
<td>.60</td>
</tr>
<tr>
<td>Salt (%)</td>
<td>3.1</td>
<td>.89</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>4.31</td>
<td>.47</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Samples were taken after brining in 6% brine
\textsuperscript{2}Standard deviation (±)
\textsuperscript{3}Total Dry Matter
\textsuperscript{4}Fat in the Dry Matter
Figure 3.1: Effect of nisin on the shelf-life of Feta cheese.

A: Total count
B: Coliform
C: Psychrotrophs
D: Lactic acid bacteria
LITERATURE CITED


Chapter 4

Development and Use of Selective Medium for Isolation of Leuconostoc Bacteria from Vegetables and Dairy Products

Noreddine Benkerroum*
and
William E. Sandine

Department of Microbiology
Oregon State University
Corvallis, OR 97331-3804

Running Title: Leuconostoc Selective Media

*Present address: Department of Food Microbiology, Institute of Agronomy and Veterinary Medicine, Hassan II, Rabat, Morocco.
ABSTRACT

A *Leuconostoc* (Leu.) selective medium (LuSM) for isolation of bacteria belonging to this genus was developed. It contained 1.0% glucose, 1.0% bacto-peptone (Difco), 0.5% yeast extract (BBL), 0.5% meat extract (Difco), 0.25% gelatin (Difco), 0.5% calcium lactate, 0.05% sorbic acid, 75 ppm sodium azide (Sigma), 0.25% sodium acetate, 0.1% (v/v) Tween 80, 15% tomato juice, 30 µg/ml vancomycin (Sigma), 0.20 µg/ml tetracycline (Serva), 0.05% cysteine hydrochloride and 1.5% agar (Difco). The medium was successfully used for the isolation and the enumeration of *Leuconostoc* bacteria in dairy products and vegetables. Of 116 strains isolated from fresh raw milk, curdled milk or various vegetables, 115 were identified as *Leuconostoc*. Among them, 89 were successfully identified to the species level as follows: *Leu. cremoris* (13.5%), *Leu. mesenteroides* subsp. *mesenteroides* (7.9%), *Leu. mesenteroides* subsp. *dextranicum* (11.2%), *Leu. mesenteroides* subsp. *paramesenteroides* (16.9%), *Leu. lactis* (10.1%) and *Leu. oenos* (40.4%). Comparative enumeration of two *Leuconostoc* species, *Leu. dextranicum* 181 and *Leu. cremoris* JLL8, on MRS agar and LuSM showed no significant difference between counts obtained on both media. Resistance to vancomycin was shown to be chromosomally encoded in one strain of *Leu. cremoris*. 
INTRODUCTION

Selective media are among the most useful tools in microbiological studies, especially in assessing the quality and safety of food products and to follow the manufacture of fermented foods. For example, detection and enumeration of some groups, species or even strains, of microorganisms is essential to assess the hygienic quality of foods prior to their release for sale or to assess the impact of specific microorganisms on a technological process. Leuconostocs are heterofermentative lactic acid bacteria (LAB) naturally occurring in milk, grass, herbage, grapes and many vegetables (29). Members of this group are widely used in dairy fermentations where they play a major role in the production of lactic acid and aroma compounds (4, 5, 23, 30). They are also associated with the malolactic fermentation of wine (15, 18) and other plant products such as pickles, green olives and sauerkraut (9, 29). Attempts have been made to find reliable media for their isolation and enumeration and both selective and differential media have been reported but, unfortunately, none has proven satisfactory. Comprehensive reviews on the Leuconostoc differential and selective media have been given by Garvie (12), Teuber and Geis (29) and Cogan (5). Differential media are mostly based on the ability of leuconostocs to utilize citrate such that they are recognized by halos developed around colonies growing on media containing insoluble calcium citrate (5, 10, 25, 34). Such differentiation is not accurate since not all leuconostocs utilize citrate; furthermore, other green plant-associated bacteria such as Lactobacillus (16) and Lc. Lactis subsp. lactis biovar. diacetylactis
also utilize citrate (24, 25). Other differential media are based on the inability of leuconostocs to reduce triphenyltetrazolium chloride (TTC) even in the presence of arginine hydrochloride, thus causing them to have white colonies. Arginine hydrolyzing bacteria, especially those of the *Lactococcus* genus, have red or pink colonies (4, 3, 32). Here *Lc. cremoris* and some lactobacilli behave in the same manner. Selective media for leuconostocs were also proposed from the use of different selective agents such as sodium azide (20), tetracycline (21, 23), and/or growth-promoting agents such as cysteine hydrochloride and tomato juice (12, 13). In all these media, further confirmation is needed and some physiological tests must be done on isolated colonies, making this procedure laborious and inappropriate for enumeration.

Chromosomally encoded vancomycin resistance in *Leuconostoc* species (8, 22, 27) as well as the sensitivity of *Lactococcus* species (8, 22) and some lactobacilli (27) to this antibiotic led us to consider its use in a selective medium. The medium (LuSM) in combination with other ingredients proved successful. Herein we report on use of the medium to isolate *Leuconostoc* species from several natural sources and food products.
MATERIALS AND METHODS

Microorganisms and Their Maintenance:

Microorganisms used in this study are listed in Table 4.1. They include bacteria and yeasts. LAB were stored in litmus milk at -20°C. Working cultures of lactococci were propagated in M17G (0.5% glucose) (28); others were propagated in MRS broth (6) using 1% inoculum and overnight incubation at 30°C. Yeasts were maintained on slants of nutrient agar (Biokar) at 4°C and propagated in nutrient broth (Biokar) using loopful inoculations and overnight incubation at 25°C.

Medium Preparation:

The basal medium contained 1.0% glucose, 1.0% bacto peptone (Difco) 0.5% yeast extract (BBL), 0.5% meat extract (Difco), 0.25% gelatin (Difco), 0.5% calcium lactate, 0.05% sorbic acid, 75 ppm sodium azide Sigma, 0.25% sodium acetate, 0.1% (v/v) Tween 80 and 1.5% of tomato juice. The tomato juice was prepared as follows: fresh tomatoes were blended in water (1:1 w/v) using a Moulinex blender. Tomato juice was then centrifuged at 8000 xg in Servall centrifuge for 15 min. The clarified tomato juice was used directly without filtration. The basal medium was sterilized by autoclaving (121°C for 15 min). The final pH of the medium varied from 5.3 to 5.8. No adjustment of pH was made unless it was below 5.0, whereupon it was adjusted with HCl to 5.5.
Stock solutions of vancomycin (Sigma), tetracycline (Serva) and cysteine hydrochloride (BDH) were prepared as follows: vancomycin and cysteine hydrochloride were dissolved in water to a final concentration of 10 mg/ml and 1 mg/ml, respectively. Tetracycline solution was prepared according to Maniatis et al. (19) by dissolving the antibiotic in ethanol to a final concentration of 5 mg/ml. All these solutions were filter sterilized by using 0.22µm pore diameter Millipore membranes and aliquots were stored at 20°C. Tetracycline solution was protected from light by covering the container with aluminum foil.

For preparation of the completed medium the basal medium was melted and tempered to about 48°C. Vancomycin, tetracycline and cysteine hydrochloride solutions were then added to final concentrations of 30µg/ml, 0.2µg/ml and 0.5 mg/ml, respectively.

**Action of Vancomycin on LAB:**

The following strains were tested for their resistance to vancomycin: *Leu. cremoris 104, Leu. cremoris CAF7, Leu. cremoris 225, Leu. cremoris 44-4, Leu. cremoris 44-4c, Leu. dextrancum 181, Lc. lactis biovar. diacetylactis BU2, Lc. lactis biovar. diacetylactis F7/22, Leu. cremoris M71, Lactobacillus bulgaricus* and Lc. cremoris AC1. Vanomycin was incorporated in MRS agar (6) at various concentrations (30, 100, 300 and 500 µg/ml), and the agar poured in sterile Petri dishes and allowed to harden and dry. An overnight culture (25µl) of the microorganism to be tested was dispensed as a
drop on the surface of MRS agar containing a given concentration of vancomycin. Plates were then incubated at 30°C and checked for growth after 2 and 4 days.

**Growth of Microorganisms on LuS.M.:**

Microorganisms listed in Table 4.1 were activated in MRS or M17G broth for LAB and nutrient broth for yeasts. Then a 25μl drop was dispensed on the surface of the new medium, previously plated and dried. Plates were then incubated at 30°C and examined daily for growth up to 4 days.

**Isolation and Identification of Microorganisms on the Experimental Medium:**

Microorganisms, 116 in number, were isolated from different products such as vegetables, milk and curdled milk. Vegetables were cut into pieces, soaked with distilled water and microorganisms isolated from the suspension. Samples of these products were serially diluted and surface plated on LuSM and then incubated at 30°C for 72 hrs. Colonies were randomly selected from plates containing uncrowded colonies and purified on MRS agar. They were then stored in litmus milk at -20°C until needed. For identification of the isolates, the four following *Leuconostoc*-identifying characteristics were tested: Morphology, catalase production, gas production from glucose, and arginine hydrolysis. Strains were identified to species level according to Garvie (11, 12). To test for
carbohydrate fermentation, the following sugars were used: lactose, sucrose, cellobiose, arabinose, galactose, fructose, xylulose, trehalose, maltose, glucose and esculin.

**Quantitative Enumeration:**

Enumeration was done on two strains: *Leu. cremoris* LL8, isolated and identified in this study, and *Leu. dextranicum* 181. These strains were grown separately in MRS broth at 30°C for 16 to 18 hours. Cultures were then serially diluted and plate-counted on LuSM. Five replications were done with *Leu. cremoris* JLL8 and 3 with *Leu. dextranicum* 181, each in duplicate. The student test was used for statistical analysis of the data.

**Plasmid Profiles:**

The procedure of Anderson and McKay (1), as modified by Wyckoff et al., (35) was followed.
RESULTS AND DISCUSSION

Resistance to Vancomycin:

The results of this study are shown in Table 4.2. All *Lactococcus* strains were sensitive to vancomycin at concentrations lower than 30 µg/ml, while all *Leuconostoc* strains were resistant to a concentration higher than 500µg/ml. Resistance of leuconostocs to vancomycin is well established (8, 14, 22, 27). Orberg and Sandine (22) and Simpson et al. (27) found members of this genus to be resistant to more than 2000 µg/ml of vancomycin. Noteworthy is that plasmid-free *Leu cremoris* 44.4C (Figure 4.1) resists the same concentration of vancomycin as the parental strain, *Leu. cremoris* 44.4, which has a 18.5 mDa plasmid (Table 4.3). This suggests that vancomycin resistance is a chromosomally encoded trait in leuconostocs. Among the 17 strains of *Leuconostoc* sp. studied by Orberg and Sandine (22), 3 without plasmids could withstand up to 2000 µg/ml of this drug. In fact, this phenotype has been used in studies as a suitable genetic marker to select for engineered leuconostocs (31). It should be emphasized however that *Leuconostoc* is not the only genus of LAB which is resistant to vancomycin; lactobacilli (8,27) and pediococci (27) were also shown to be resistant to this antibiotic, *L. bulgaricus* could tolerate only up to 100 µg/ml. Nonetheless, unlike *Leuconostoc*, this phenotype is not common in lactobacilli. Eighty percent of 20 *Lactococcus* strains tested by Vascovo et al. (33) were found sensitive. Simpson et al. (27) showed that homofermentative lactobacilli in the thermobacterium group were vancomycin-sensitive
while heterofermentative lactobacilli were resistant. In contrast, the sensitivity of lactococci to vancomycin at concentrations less than 10 \( \mu g/ml \) is well documented (8, 22, 27). Simpson et al. (27) recommended that 50 \( \mu g/ml \) of vancomycin should be used in selective media.

**Selectivity of the LuSM:**

*Lactococcus* sp., *Lb. bulgaricus* and the yeasts tested were all completely inhibited. Although *Lb. bulgaricus* grew on vancomycin containing MRS, it did not on the LuSM, suggesting that other ingredients of the medium were inhibitory for that strain.

**Isolation and Identification:**

All isolated strains but one were Gram-positive, catalase-negative gas producing cocci which did not hydrolyse arginine. They were then regarded as leuconostocs. The exception did not produce gas and formed cocci assembled in tetrads, typical of pediococci. Eighty-nine Leuconostoc strains were identified to the species level (Table 4.4). Results showed that the most common Leuconostoc species represented was *Leu. oenos* (36% of the identified isolates), which may have been due to its stimulation by cysteine hydrochloride (12). No lactobacilli were found among the isolated strains, despite the fact that many of them have been reported to be resistant to vancomycin and that they have the same natural habitat as leuconostocs (8, 12). The combination of many agents inhibitory to most LAB other than
*Leuconostoc* spp. and the presence of stimulatory agents to some species of this genus would lead to its outgrowth over the rest of LAB. Sodium azide was reported to be inhibitory to most LAB including lactobacilli (17) but not leuconostocs (20) or *Lc. diacetylactis* (Sandine, unpublished data); therefore, this compound was used by Mayeux et al. (20) as a basis for the formulation of a *Leuconostoc* selective medium. Tetracycline was also shown to be inhibitory to lactococci at low concentrations, less than 10 μg/ml (21). No data are available to our knowledge on the effect of this antibiotic on lactobacilli or pediococci; nonetheless, it has been used in media selective for leuconostocs (21, 23).

The major contribution of vancomycin in this medium is to achieve a total inhibition of lactococci and to inhibit some lactobacilli as well, mainly the homofermentatives (27). The latter group of microorganisms is also inhibited by sorbic acid. Their sensitivity to sorbate increases at pH values below 6.3 (27). In contrast, sorbic acid was shown to stimulate growth of leuconostocs (7). It is also inhibitory to yeasts (2).

**Quantitative Determination:**

No significant difference (P <0.05) was found between counts on MRS and LuSM for both strains (Table 4.5). In the case of *Leu. dextranicum* 181, suitable colony size on the new medium was obtained after 3 to 5 days of incubation, while on MRS 24 hours of incubation were sufficient. In view of these results, LuSM would be
recommended for the isolation and the enumeration of leuconostocs in mixed starter cultures or in fermented products containing a heterogeneous flora.
Table 4.1: Bacterial and yeast strains used in the present study and their origin.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
</tr>
<tr>
<td><em>Lc. cremoris</em> AC1</td>
<td>FDRC</td>
</tr>
<tr>
<td><em>Leu. diacetylactis</em> F7/22</td>
<td>FDRC</td>
</tr>
<tr>
<td><em>Leu. cremoris</em> 225</td>
<td>OSU</td>
</tr>
<tr>
<td><em>Lc. lactis</em> 7962</td>
<td>OSU</td>
</tr>
<tr>
<td><em>Lc. lactis</em> LB11</td>
<td>previous study</td>
</tr>
<tr>
<td><em>Leu. cremoris</em> 44-4</td>
<td>(26)</td>
</tr>
<tr>
<td><em>Leu. cremoris</em> 44-4C*</td>
<td>(26)</td>
</tr>
<tr>
<td><em>Leu. cremoris</em> CAF-7</td>
<td>OSU</td>
</tr>
<tr>
<td><em>Leu. cremoris</em> 104</td>
<td>OSU</td>
</tr>
<tr>
<td><em>Lc. diacetylactis</em> BU2</td>
<td>FDRC</td>
</tr>
<tr>
<td><em>E. coli</em> V517</td>
<td>OSU</td>
</tr>
<tr>
<td><em>Micrococcus flavus</em></td>
<td>OSU</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>IAV</td>
</tr>
<tr>
<td><em>Leu. cremoris</em> M71</td>
<td>FDRC</td>
</tr>
<tr>
<td><em>Lb. bulgaricus</em></td>
<td>Yogurt starter (Rediset)</td>
</tr>
<tr>
<td><em>Str. thermophilus</em></td>
<td>Yogurt starter (Rediset)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Yeasts</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>IAV</td>
</tr>
<tr>
<td><em>Candida milleri</em></td>
<td>IAV</td>
</tr>
</tbody>
</table>

FDRC = Federal Dairy Research Center (Kiel, Germany)
OSU = Oregon State University (USA)
IAV = Institute of Agronomy & Veterinary Medicine (Rabat, Morocco).
Cured derivative of *Leu. cremoris* 44-4
Table 4.2: Plasmid profiles of cultures used.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Molecular weight (Kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> V517</td>
<td>56, 7.6, 5.8, 5.3, 4.1, 3.2, 28, 2.1</td>
</tr>
<tr>
<td><em>Leu. cremoris</em> 44-4</td>
<td>18.5</td>
</tr>
<tr>
<td><em>Leu. cremoris</em> 44-4C</td>
<td>None</td>
</tr>
</tbody>
</table>
Table 4.3: Susceptibility of cultures used to vancomycin:

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu. cremoris 104</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Leu. cremoris 225</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Leu. cremoris CAF7</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Leu. cremoris 44-4</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Leu. cremoris 44-4C</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Leu. cremoris M71</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Leu. dextranicum 181</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Lb. bulgaricus</td>
<td>&lt;100</td>
</tr>
<tr>
<td>Lc. diacetylactis BU2</td>
<td>&lt;30</td>
</tr>
<tr>
<td>Lc. diacetylactis F7/22</td>
<td>&lt;30</td>
</tr>
<tr>
<td>Lc. cremoris ACI</td>
<td>&lt;30</td>
</tr>
</tbody>
</table>
Table 4.4: Identification of *Leuconostoc* strains used in this study.

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>Number</th>
<th>Percent (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Leu. cremoris</em></td>
<td>12</td>
<td>13.5</td>
</tr>
<tr>
<td><em>Leu. dextranicum</em></td>
<td>10</td>
<td>11.2</td>
</tr>
<tr>
<td><em>Leu. mesenteroides</em></td>
<td>07</td>
<td>7.9</td>
</tr>
<tr>
<td><em>Leu. paramesentroides</em></td>
<td>15</td>
<td>16.9</td>
</tr>
<tr>
<td><em>Leu. lactis</em></td>
<td>09</td>
<td>10.1</td>
</tr>
<tr>
<td><em>Leu. oenos</em></td>
<td>36</td>
<td>40.4</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>89</td>
<td>100</td>
</tr>
</tbody>
</table>
Table 4.5: Enumeration of *Leu. dextranicum* 181 and *Leu. cremoris* JLL8 on MRS agar and on LuSM.

<table>
<thead>
<tr>
<th></th>
<th>Leu. cremoris JLL8*</th>
<th>Leu dextranicum 181**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MRS</td>
<td>LuSM</td>
</tr>
<tr>
<td>Counts (CFU/ml)</td>
<td>$1.96 \times 10^8$</td>
<td>$1.8 \times 10^8$</td>
</tr>
<tr>
<td>S. D.</td>
<td>$1.7 \times 10^8$</td>
<td>$1.3 \times 10^8$</td>
</tr>
</tbody>
</table>

*values are means of 5 determination in duplicates
**values are means of 3 determinations in duplicates
S.D. = Standard deviation
Figure 4.1: Plasmid profiles of *Leuconostoc cremoris* 44-4 and its cured derivative.

Lane A: *E. coli* V517 (Standard)
Lane B: *Leuconostoc cremoris* 44-4
Lane C: *Leuconostoc cremoris* 44-4C
LITERATURE CITED


BIBLIOGRAPHY


Orla-Jensen. Cited by Kandler.


