AN ABSTRACT OF THE THESIS OF

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Title: Effect of Selected Lactic Acid Bacteria On The Growth Of Food-borne Pathogens And Spoilage Microorganisms in Raw Milk And Milk Products

Abstract Approved: _____
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Several lactic acid bacteria (LAB) of the *Lactococcus, Lactobacillus, Leuconostoc* and *Pediococcus* genera were screened for inhibition of food-borne pathogens and spoilage microorganisms in raw milk and dairy products. *Listeria monocytogenes* was killed by *Lactococcus lactis* subsp. *lactis* and *Pediococcus pentosaceus* due to their production of bacteriocin-type inhibitors. *Staphylococcus aureus* was not able to grow in raw milk at temperatures below 5°C even without LAB being present. Gram negative *Salmonella enteritidis, Salmonella typhimurium* and *Escherichia coli,* along with spoilage bacteria of the genus *Pseudomonas* were dramatically inhibited by a *Lactobacillus* species , designated AS-1, in raw and pasteurized milk as well as in cottage cheese. However, other LAB were not able to inhibit these organisms. *Lactobacillus* AS-1, did not produce hydrogen peroxide but carbon dioxide was produced. The AS-1 strain was a gram positive coccobacillus, catalase and oxidase

negative and produced DL-lactic acid. It deaminated arginine and grew over a temperature range of 5°C to 45°C. It was also able to ferment glucose, galactose, fructose and lactose in addition to 17 other carbohydrates. High numbers (10^7 CFU/ml) of AS-1 were required to obtain complete inhibition of gram negative bacteria. A selective medium (ASLM) for *Listeria monocytogenes* was developed to follow the fate of this particular pathogen in association with LAB in raw milk; other selective media were not able to inhibit the growth of background flora of raw milk. ASLM was superior to four other media in allowing only the growth of the target pathogen. For the *Lactococcus* genus, a selective and differential agar medium (Alsan) was formulated to selectively allow growth of *Lactococcus* spp. and to differentiate between *Lactococcus lactis* subsp. *lactis* and the biovariety *diacetylactis,* based on citrate utilization.

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TABLE OF CONTENTS

LIST OF FIGURES

FIGURES PAGE

FIGURES PAGE

LIST OF TABLES

TABLES PAGE

EFFECT OF SELECTED LACTIC ACID BACTERIA ON THE GROWTH OF FOOD-BORNE PATHOGENS AND SPOILAGE MICROORGANISMS IN RAW MILK AND MILK PRODUCTS

INTRODUCTION

Lactic acid bacteria (LAB) are a diverse group of bacteria consisting of the *Streptococcus, Leuconostoc, Pediococcus* and *Lactobacillus* genera (6, 16, 19). Recently, a new genus was established to replace group N *Streptococcus* and is known as *Lactococcus* (12). This new genus includes the species *lactis, garviae, plantarum raffinolactis*, and *piscium* (12, 21) as well as several subspecies and one biovariety.

LAB are gram-positive rod, coccus and coccobacillary shaped bacteria (4, 6, 15). They are catalase and oxidase negative and consist of two different groups based on fermentation end products. Homofermentative LAB produce mainly lactic acid from glucose while the heterofermentative types produce acetate , ethanol and CO_2 in addition to lactic acid (13, 16). Milk and dairy products, vegetables, plants and the intestinal tract of humans and animals are natural reservoirs for LAB. A new genus, *Carnobacterium*, was separated out from the *Lactobacillus* genus based on certain unique characteristics (4, 10); they are commonly found in meat.

Ancient civilizations unknowingly relied on LAB for natural fermentations of milk, vegetables, meat and fruit. Centuries later they became well characterized and currently, they are used for production of fermented foods under controlled conditions (18, 19, 20). Fermentations by LAB result in lactic acid production which causes a decrease in pH. Their presence in foods plays a major role in inhibiting the growth of food spoilage and pathogenic microorganisms. Also, their metabolites in some food products improve texture and flavor. LAB also are used to extend food shelf-life and to inhibit pathogens (1, 14). In addition to lactic acid, LAB may produce acetic acid, hydrogen peroxide, diacetyl, carbon dioxide

and bacteriocins, each of which may be inhibitory to undesirable microorganisms in various foods.

Numerous research works have described different inhibitory substances produced by LAB (5, 9, 11). Nisin, a bacteriocin produced by certain strains of *Lactococcus lactis* subsp. *lactis*, is inhibitory to certain gram-positive bacteria such as *Listeria monocytogenes* (3, 14). This bacteriocin also will inhibit some gram-negative bacteria when its activity is potentiated by chelating agents (17). Studies at the molecular level have indicated that LAB contain plasmids, some of which may encode for various functions such as bacteriocin production and carbohydrate fermentation (2, 8, 9) In view of this, it is likely that different applications of inhibitor-producing LAB in food preservation will be made in the future. This thesis concerns one such application wherein LAB were screened for their ability to preserve refrigerated raw milk from quality degredation due to growth of spoilage and pathogenic bacteria. Thus initial efforts in this research involved screening different LAB for use in this regard. Because of the great concern about the presence of *L. monocytogenes* in the food supply, this was the first pathogen we attempted to inhibit using LAB. Early in this effort, it became evident that none of the available selective media for this organism was suitable for use in this research. Therefore, time was taken to attempt to develop such a medium. Since this effort was successful, a separate chapter provides details on its development. Later in this study, the need for another selective media became apparent to accurately determine the fate of *Lactococcus* organisms when added to raw milk along with other bacteria.

This thesis is presented in six chapters and in the order that was followed in carrying out the research wherein the overall objective was to identify LAB which would lengthened the shelf-life of raw and pasteurized milks.

Chapter 1: Antagonistic Effect of Lactic Acid Bacteria on Foodborne Pathogens in Raw Milk During Cold Storage

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CHAPTER 1

Antagonistic Effect of Lactic Acid Bacteria on Foodborne Pathogens in Raw Milk During Cold Storage[a]

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ABSTRACT

Raw milk was inoculated with different lactic acid bacteria (LAB) representing the *Lactococcus, Lactobacillus* and *Leuconostoc* genera. The milk then was artificially contaminated with about 10^2 CFU/ml of *Listeria monocytogenes, Staphylococcus aureus, Salmonella* spp. or *Escherichia coli*, followed by cold storage (4.4°C); control raw milk contained no added LAB. *Listeria monocytogenes* was eliminated by *Lactococcus lactis* subsp. *lactis* and *Pediococcus pentosaceus* whereas *Salmonella* and *E. coli* as well as other gram-negative bacteria were inhibited by a *Lactobacillus* isolate (AS-1). *Staphylococcus aureus* did not survive the cold temperature even without LAB. Inhibition of gram-negative bacteria by *Lactobacillus* AS-1 was not caused by lactic acid or hydrogen peroxide production. Temperature abuse (>7.5°C) resulted in an increase of *Listeria monocytogenes* by more than 99% whereas gram-negative bacteria reached more than 10^6 CFU/ml at 5°C in one week.

INTRODUCTION

In many parts of the world, time elapses before raw milk is pasteurized. This is due to transportation of raw milk to processing facilities, shortage of milk supplies to large dairy plants and scheduling problems in creameries. During this time delay and prior to thermal processing, contaminating microorganisms, including health-hazard pathogens, can grow and multiply even at refrigeration temperatures (4-5°C). Psychrotrophic bacteria such as *Pseudomonas* spp., *Clostridium botulinum* type E and *Listeria monocytogenes* are able to grow and multiply at temperatures as low as 3°C and therefore refrigeration is incapable of restricting their growth (41). Several methods are being used to extend the shelf-life of raw milk such as thermization (65°C for 15 sec.) (24, 31), CO_2 injection (13, 28), activation of the lactoperoxidase system (17, 18, 38, 55) and addition of lactic acid bacteria (LAB) (12, 13, 28, 32, 55). The addition of LAB is intended to inhibit the growth of *Pseudomonas* and related genera which degrade milk protein and fat and hence affect the quality of milk and products made therefrom. Several authors have pointed out the effects of these types of bacteria on milk quality (8, 14, 35, 52). In this regard, improving the quality of pasteurized milk and skim-milk powder made from raw milk stored at 2°C, rather than 6°C, was a result of temperature inhibition of psychrotrophs and their thermostable enzymes (26, 27). Regulations in the U.S.A. require that producer raw milk should have ≤ 100,000 CFU/ml and ≤ 300,000 CFU/ml for comingled raw milk. For pasteurized milk, not more than 20,000 CFU/ml are allowed. Bacterial count standards for pathogenic bacteria in raw milk do not exist. Inhibition of *Pseudomonas* spp. in raw milk by LAB is intended to improve quality of milk products and increase cheese yield. The degree of inhibition of *Pseudomonas* sp. by LAB has been reported in Juffs and Babel (32) to decrease as the number of these spoilage bacteria increase over long periods of cold storage (32). However, they provided no data regarding the number of LAB added to milk initially. Other LAB were found to be ineffective in retarding growth of spoilage organisms in raw milk (54), whereas in other cases, it was necessary for LAB to drop the pH

to as low as 4.8 in order to achieve inhibition (28, 54); this low pH makes the process of no practical use since the acidified milk would be unstable to further processing. Inhibitory activity of LAB on *Pseudomonas* sp. was reported to be related mainly to lactic acid production (13, 28) but other reports have noted the marginal effect of lactic acid on psychrotrophic bacteria (32, 45). Methods used to enumerate *Pseudomonas* sp. (surface plating vs. agar droplet method) in LAB-inoculated raw milk have been reported to influence the degree of inhibition observed by LAB (12).

The effects of LAB on pathogens in raw milk have not been documented in the literature despite the fact that pathogens such as *Salmonella* and *Listeria monocytogenes* have been repeatedly isolated from raw milk supplies (5, 7, 10, 15, 19, 25, 37, 47, 51). Illnesses caused by foodborne pathogens result in medical costs as well as loss of productivity (4, 36). It is to the interest to both consumers and the dairy industry to eliminate pathogens from raw milk before pasteurization. Recent reports have noted the growth stimulatory effect of some spoilage bacteria such as *Pseudomonas* on *Listeria monocytogenes* in raw milk (20, 39). The objective of this research work was to examine the possible beneficial effects of adding LAB to raw milk by inhibiting food spoilage and pathogenic bacteria.

MATERIALS AND METHODS

Raw Milk (RM):

Fresh RM was obtained from Oregon State University Dairy Farm and immediately refrigerated at 4.4°C. Standard plate count of RM was conducted at zero time and over one week period, using Plate Count Agar (PCA) and incubation at 32°C for 48 hours.

Bacterial Cultures:

LAB (Table 1.1) were activated in M17 (Baltimore Biological Laboratory, Baltimore, MD) or MRS broth (Difco Laboratories, Detroit, MI) at 30°C for mesophiles and 37°C for thermophiles for 24 hours. Pathogenic organisms (Table 2) were propagated in BHI broth (Difco) at 37°C for 24 hours. Each pathogen was serially diluted in 0.1% peptone water (sterile) and appropriate dilutions used for RM inoculation.

Associative Growth of LAB and Pathogens:

Refrigerated RM was inoculated with approximately 1 x 10^2 CFU/ml of a particular pathogen. The CFU/ml was determined at zero time and over a one week period of storage at 4.4°C. Meanwhile, each LAB was added to RM at (10^7-10^8 CFU/ml) from active broth cultures. During inoculation, RM was kept on ice to insure that the temperature was not raising above 4.4°C. Furthermore, control RM (without either LAB or pathogen or with a pathogen only) was used. After inoculation, both samples and controls were kept at the desired temperature for a one-week period.

Microbial Analysis:

a) Pathogens: Recovery of each pathogen (spread plate technique) from RM (both samples and control) was carried out using two selective agar media (Table 1.2).

b) Cultures of LAB: The growth profile of LAB in RM during cold storage was not monitored because of the unavailability of satisfactory selective media for their ennumeration. However, *Lactococcus* genus was monitored during refrigeration using a selective and differential medium developed for their isolation (3). The spiral plating system (Spiral System™, Cincinnati, OH) was used to recover *Lactococcus* spp. from raw milk.

c) Standard Plate Count: The level of aerobic organisms of RM was determined during incubation at 4.4°C for seven days. Numbers present were determined using PCA and incubation at 32°C for 48 hours using the Model D Spiral Plater.

Effect of pH and Lactic Acid:

Inhibition of gram-negative bacteria present naturally in RM as a result of pH reduction and lactic acid production by *Lactobacillus* AS-1 was investigated. RM was inoculated with 3% *Lactobacillus* AS-1 (10^7 CFU/ml) and a control RM (without *Lactobacillus* AS-1) was used as well. Both samples and controls were stored at 4.4°C for a week. Enumeration of gram negative bacteria other than *Pseudomonas* spp. was done using MacConkey agar (Difco), a selective medium for enterics. The pH of RM with or without *Lactobacillus* AS-1 was measured during cold storage. Another control RM was adjusted to the same pH as that of RM with AS-1 with a 10% solution of DL lactic acid (sterile). Eight hours after addition of acid and pH adjustments at 4.4°C, the CFU/ml of gram-negative bacteria (enterics) was carried out in RM + AS-1, RM (control) and RM adjusted by adding lactic acid. The Spiral System™ plating was used and plates incubated at 37°C for 48 hours. At least three trials were conducted and the mean of all trials were used.

RESULTS AND DISCUSSION

Survival of Pathogens in Association with LAB

As seen in Table 1.3, LM (strains Scott A and ATCC 7644) were only eliminated (<10 CFU/ml) by *Lactococcus lactis* subsp. *lactis* ATCC 7962 and *Pediococcus pentosaceus* (*cerevisiae*). In this regard, several authors have reported inhibition of this pathogen by nisin, a bacteriocin produced by *Lactococcus lactis* subsp. *lactis* (29, 34, 53). A recent study by Stevens et al., (49) showed expansion of the inhibitory activity of nisin to gram negative bacteria by including chelating agents with nisin. Furthermore, *Pediococcus* was found inhibitory against LM (1) and different reports have characterized inhibitors produced by several pediococci (6, 30, 40). Other LAB maintained the number of LM below 10^2 during cold storage (Table 1.3). The CFU/ml of LM in RM without LAB increased slightly (<0.5 log) during refrigeration for seven days (Table 1.3) since it is a psychrotrophic bacterium and cold enrichment usually is used for its isolation. In other food systems such as meat, low levels of LM did not increase over two weeks at 4°C (33). The survival of low numbers of LM in RM at 4.4°C indicated the ineffectiveness of the natural inhibitors of RM. Similar results were reported for the activated lactoperoxidase system (LPS) of RM on inhibiting LM (16, 46). On the other hand, Gaya et al, (22), pointed out that LPS inhibition of LM was dependent on temperature, length of incubation and the strain used. However, LPS has been found to be inactive against gram-positive bacteria (46). An attempt was made during the present study to inactivate LPS of RM by raising the pH to 7.5 with NaOH (7) followed by addition of LM and then refrigeration for a week. There were no differences (data not shown) in the count of this pathogen in RM with or without adjusting the pH to 7.5. However, temperature increases, whatever the cause, could present a high risk situation. As a matter of fact, LM increased by more than a hundred-fold in less than a week in both sterilized milk and RM without LAB when the temperature increased to 8°C due to electrical problems in the thermostat of the refrigerator. Another factor which may influence growth of LM in milk is types of other bacteria

present. In this regard, it has been found that *Pseudomonas* and related genera are stimulatory to LM (20, 39).

In the present research, two LM selective agar media were used (Table 1.2). Difficulties were encountered using Oxford agar but not ASLM (2), especially when esculin positive bacteria such as gram-positive cocci were present since they grew on Oxford agar causing confusion in LM identification. Furthermore, a gram-negative, esculin-positive bacterium was isolated on Oxford agar when the pH of RM was adjusted to 6 with concentrated hydrochloric acid and stored at 4.4°C for more than two days. The same bacterium, however, was not isolated when the pH of RM was unchanged after a week of incubation. Among all the lactic type bacteria used, only *Propionibacterium shermanii* grew heavily in Oxford agar but it appeared as esculin negative. While LM grew slightly during refrigeration (4.4°C) without LAB, *Staphylococcus aureus* (2 strains) was not able to survive and the count was below the detectable level (<10 CFU/ml) after seven days at 4.4°C in both RM and sterilized milk (Table 1.4). This pathogen also was antagonized when grown in association with *Lactobacillus bulgaricus* for four days at 4.4°C (Table 1.4). Thus temperature used in this study likely is below the minimum temperature for its growth. The best medium for recovering *Staphylococcus aureus* from RM, especially at zero time, was Baird-Parker agar since *Staphylococcus* 110 medium was inhibitory to the low level of this pathogen used. In fact, in a separate study (unpublished data) both Oxford and MVJ (11) were comparable to Baird-Parker agar in recovering uninjured and heat-injured cells of *Staphylococcus aureus Salmonella enteritidis* and *Salmonella typhimurium* neither died nor increased in numbers above 10^2 CFU/ml in RM over the week period at 4.4°C (Table 1.5). However, other gram-negative bacteria present naturally in RM reached more than 10^5 CFU/ml (99.9% increase) upon cold storage for a week with or without the presence of LAB (Table 1.5). This group was found to be made up of coliform, *Pseudomonas* and unidentified genera when recovered in Hektone-enteric agar (Difco). *Lactobacillus* AS-1 was the only LAB which kept these organisms and *Salmonella* species below the detected level (<10 CFU/ml) (Table 1.5). There

have been some reports on the inhibitory activity of the LPS of RM as well as hydrogen peroxide (H_2O_2) produced by certain lactobacilli on gram-negative bacteria (9, 42, 48). Our results regarding inhibition of gram-negative bacteria by the LPS of RM and H_2O_2 suggest otherwise. These bacteria grew rapidly in RM with or without LAB except when *Lactobacillus* AS-1 was present. This organism inhibited them, but did not produce H_2O_2 when tested in a diagnostic medium developed by Fontaine and Taylor-Robinson, (21). For further information on the natural inhibitors of RM, consult Ekstrand, (17) and Reiter, (44).

Lactobacillus AS-1 added to RM provided >99% inhibition of *E. coli* V517 and other naturally-occurring, gram-negative bacteria after seven days at 4.4°C (Table 1.6) and Figure 1.1. Adding catalase to RM with this particular LAB did not counteract the antagonistic activity towards gram-negative bacteria as enumerated in MacConkey agar, suggesting the absence of H_2O_2 as a contributor to the inhibition.

pH and Lactic Acid Effects

RM adjusted to the same pH with lactic acid as milk containing *Lactobacillus* AS-1 (1×10^7 CFU/ml) had the same microbial load (gram-negative enterics) as the control RM when neither were inoculated with *Lactobacillus* AS-1 nor adjusted to the same pH (Table 1.7). Similarly, Ross, (45) and Reinheimer et al., (43) pointed out the minimal effect of lactic acid and pH reduction below 6 on milk spoilage organisms. On the other hand, Champagne et al., (13) and Griffiths et al., (28), stated that the effect of lactic acid produced by certain LAB on *Pseudomonas* spp. was apparent only at pH values below 6.The same results were obtained when sodium acetate was added to acheive the same pH which strain AS-1 produced in raw milk , indicating that acetate at pH 6 has a minimum inhibitory activity in refrigerated raw milk at this pH. It is well known that acetic acid at a pH near or below its pKa (4.8) is inhibitory to many microorganisms. However, at pH 6.0 about 93% of this acid is ionized, in which form it can not penetrate bacterial cells. Other workers have reported on the ineffectiveness of metabolites of LAB (23, 40)

in inhibiting gram-negative bacteria. In our study, *Lactobacillus* AS-1 produced only enough acid to drop the pH by 0.5 unit over a week of cold storage (5°C). Inhibition of gram-negative bacteria started when the pH fell only to 6.4, still likely too high for there to be sufficient concentrations of acids present to be inhibitory.

It is not unusual to find $>10^6$ CFU/ml gram-negative bacteria in pasteurized market milk products, even though the Federal Standard is <20,000/ml. This speaks to the constant vigil necessary in processing plants to minimize psychrotrophic post-pasteurization contam-ination and explains why researchers continue to seek new methods to inhibit these bacteria in dairy products.

Application of Adding *Lactobacillus* AS-1 to RM

Strain AS-1 was added to fresh RM at 1 x 10^7 CFU/ml at zero time followed by incubation at 4.4°C for six days. Pasteurization in a water bath at 63°C for 30 min. followed by cooling did not result in any undesirable changes occurring in the pasteurized milk as to taste, color or coagulation. This makes this particular strain useful in inhibiting gram-negative bacteria in good quality RM when added after milking to the refrigerated (4.4°C) milk and which may be held for up to a week before pasteurization.

Growth of LAB and Background Flora During Refrigeration

The level of *Lactococcus* genus in RM during cold storage was almost constant, (10^7 CFU/ml) as indicated by the count in Alsan medium. Similarly, Champagne et al., (13) found no growth of LAB in refrigerated milk when a non-selective medium, M17, was used. On the other hand, LAB grew in RM during cold storage as reported by Griffiths et al., (28). To assess the growth of LAB, a selective media for their isolation will be required, but none exist today.

Standard plate count of RM increased from $<10^2$ to $>10^6$ CFU/ml over a week of cold storage. This high microbial load will certainly be detrimental to RM quality and the possibility of growth of

psychrotrophic pathogens such as *Listeria, E. coli, Aeromonas* and *Yersenia* should be kept in mind. Reservoirs of these bacteria in raw milk also pose a threat to the possibility of post-pasteurization contamination in the dairy processing environment.

Table 1.1. Lactic Acid Bacteria (LAB) screened during this study

Organism	Strain
Lactococcus lactis subsp. *lactis*	ATCC 7962
	F2D2
biovar. *diacetylactis*	18-16
	DRC1
subsp. *cremoris*	107/6
	205
Lactobacillus bulgaricus	Y
Lactobacillus isolate	AS-1
Leuconostoc cremoris	104
Pediococcus pentosaceus	A
Streptococcus thermophilus	CR5
Propionibacterium shermanii[a]	ATCC 9616

[a] = non LAB

Table 1.2. Pathogens studied and selective agar media used for their recovery from raw milk.

Pathogen	Strain	Selective Agar[a]
Salmonella typhimurium	ATCC13311	Bismuth Sulfite and
Salmonella enteritidis	ATCC4931	Hekton-enteric agar
Staphylococcus aureus	OSU ATCC13565	Baird-Parker and Staph. 110 medium
Listeria monocytogenes	ATCC7644 Scott A	Oxford agar and ASLM
Escherichia coli V517		Macconkey agar and
E. coli	ATCC23716	EMB agar

[a]All were Difco products except those used for *L. monocytogenes*

Table 1.3. Behavior of *Listeria monocytogenes* (strain Scott A and ATCC 7644) in the presence of LAB during cold storage of raw milk at 4.4°C.

	Log_{10} CFU/ml after storage for: 4 days	7 days
Control raw milk	No growth[a]	No growth
+ *Listeria monocytogenes* (LM)	2.01	2.25
+LM, *P. pentosaceus* or *L. lactis* subsp. *lactis*	<1	<1
+LM, other LAB	<2	<2

[a]No LM was recovered from raw milk (control) using Oxford and ASLM selective media.

Table 1.4. Effect of LAB on *Staphylococcus aureus* in raw milk during storage at 4.4°C.

	Log_{10} CFU/ml after storage for: 4 days	7 days
Control raw milk	<1	<1
+ *Staphylococcus aureus*	2.2	<1
+ *Staphylococcus aureus* and *Lactobacillus bulgaricus* Y	<1	<1

Table 1.5. Comparison of $\log_{10}$ CFU/ml of *Salmonella* spp. in the presence or absence of LAB during one week of cold storage at 4.4°C.

	0 days		4 days		7 days	
	BS	HE[a]	BS	HE	BS	HE
Control raw milk	<1	<1	<2	>3	<2	>5
+ *Salmonella enteritids* or *S. typhimurium*	2.1	<1	<2	4	<2	>5
+ *Salmonella* (either species) and *Lactobacillus* AS-1			<1	<1	<1	<1
+ *Salmonella* (either species) and any other LAB			<2	>3	<2	>5

BS = Bismuth sulfite agar
HE = Hekton-enteric agar
[a] = The $\log_{10}$ CFU/ml in HE represented other gram-negative bacteria in addition to *Salmonella* spp.

Table 1.6. Effect of *Lactobacillus* AS-1 on *E. coli* and other gram-negative bacteria present naturally in raw milk during incubation at 4.4°C for seven days.

	CFU/ml recovered on MacConkey agar or EMB
Cold storage for:	
0 day (control raw milk with *E. coli*)	1×10^3
3 days	
control	1.4×10^4
+*Lactobacillus* AS-1	8.5×10^3 (40%)[a]
7 days	
control	$>1 \times 10^7$
+*Lactobacillus* AS-1	$<1 \times 10^3$ (>99%)

[a] Values in parenthesis represents percentage inhibition by *Lactobacillus* AS-1 in comparison with the corresponding control.

Table 1.7. The antagonistic activity of *Lactobacillus* AS-1 added to raw milk on gram-negative bacteria during cold storage in comparison with the inhibition by DL-lactic acid (10% solution) added to adjust the pH.

	CFU/ml[a] after incubation at 4.4°C for:	
	5 days	7 days
Control raw milk	$>10^5$	$>1 \times 10^7$
Control raw milk with DL-lactic acid	$>10^5$	$>1 \times 10^7$
Control raw milk inoculated with *Lactobacillus* AS-1	$<4 \times 10^2$	$<4 \times 10^2$

[a]CFU/ml of raw milk was done using MacConkey agar as a selective medium for gram-negative bacteria. CFU/ml at zero time was $<4 \times 10^2$ using the spiral system.

Figure 1.1: Growth of *E. coli* and other natural gram-negative bacterial flora ($>10^7$ CFU/ml) in the absence of *Lactobacillus* AS-1 for 7 days at 50°C (left). Inhibition of these bacteria ($<10^2$ CFU/ml) by AS-1 under the same conditions (right). MacConkey agar was the selective medium for gram negative bacteria (enterics).

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CHAPTER 2

Growth Inhibition of *Pseudomonas* spp. in Refrigerated Raw and Pasteurized Milks and Cottage Cheese by *Lactobacillus* AS-1

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ABSTRACT

Lactobacillus AS-1 was tested for inhibitory activity against *Pseudomonas* spp. in raw and pasteurized milk. Natural milk background flora as well as gram negative bacteria added to milk were dramatically inhibited by the AS-1 strain in comparison to control milk when samples were held over a week at 5°C. Control milk without added strain AS-1 supported growth of the natural flora to $>10^7$ CFU/ml while milk with added AS-1 restricted growth of the natural flora or added pseudomonads to $<10^2$ CFU/ml. 10^7 CFU/ml of strain AS-1 in milk was required to obtain this inhibition. Hydrogen peroxide was not produced by strain AS-1 and lactic acid was not the sole compound responsible for inhibition. *Lactobacillis* AS-1 was a vigorous CO_2 producer as a result of lactose fermentation, but, suprisingly, the pH of raw milk inoculated with 10^7 CFU/ml of this bacterium reached only as low as 6.1 when held for a week at 4.4°C. *Lactobacillus confusus*, *Lactobacillus bulgaricus* and *Lactobacillus fermentum* added to raw milk at the same populations failed to inhibit the growth of gram negative bacteria. Therefore, the AS-1 strain was unique in this inhibitory property which distinguished it from other lactobacilli.

INTRODUCTION

Psychrotrophs are a group of microorganisms which can grow reasonably well at refrigeration temperatures (2-5°C.) regardless of their optimum growth temperature. *Pseudomonas* spp., some *Bacillus* spp. and even pathogens such as *Listeria monocytogenes* are included in this group. The growth of spoilage psychrotrophs in milk and milk products is detrimental to product quality. Psychrotrophic organisms of the genus *Pseudomonas* produce proteases and lipases which may survive pasteurization and thus shorten the shelf-life of dairy products (6, 12, 17 , 18, 24, 26). Raw milk is usually held for a few days before processing and during this time psychrotrophic microorganisms proliferate and reduce overall milk quality. In the same manner, post-processing contamination of pasteurized milk adversely affects the shelf-life. Furthermore, growth of *Pseudomonas* spp. in milk has been shown to stimulate *Listeria monocytogenes* (14, 25). The United States Public Milk Ordinance Code (1987) requires that. raw milk producers deliver milk to haulers containing no more than 10^5 CFU/ml and that comingled milk from several producers contain no more than 3 x 10^5 CFU/ml; pasteurized milk for human consumption must not contain >2 x 10^4 CFU/ml.

Several approaches, other than cleaning and sanitation, have been used to restrain the growth of milk spoilage microorganisms, especially psychrotrophs. These include adequate refrigeration (<5°C) (4, 17 ,18), thermization at 65°C for 15 seconds (20), injection of CO_2 (23, 29), activation of the lactoperoxidase system by H_2O_2 (16, 34), inoculating with lactic acid bacteria (2, 8, 9, 19, 21, 28, 30, 33) and the addition of sorbate (16). Adding lactic acid bacteria (LAB) to refrigerated milk could be another acceptable approach since it is known that these organisms inhibit undesireable organisms and advantage of this, often unknowningly, has been taken from even before the advent of refrigeration and still is relied upon in some parts of the world. With this possibility in mind, the present study was undertaken to examine the effect on microbial growth of adding a *Lactobacillus* isolate to raw and pasteurized milks as well as to

cottage cheese. Also, the mechanism by which this bacterium inhibits growth of *Pseudomonas* spp. is considered.

MATERIALS AND METHODS

Bacterial Cultures:

Lactobacillus AS-1 as well as other cultures were kept frozen at -20°C in 11% non-fat dry milk (NDM) containing 60% glycerol (2 ml NDM + 0.5 ml glycerol) before activation. LAB were propagated in MRS broth (Difco) or 11% NDM at 30°C for 18-24 hours whereas *Pseudomonas* spp. were activated in BHI broth (Difco). *Pseudomonas putida* W and ATCC 12633, *P. nigrificans* and *P. putrefaciens* were screened for inhibition by AS-1. Other cultures used for comparative study were *Lactobacillus bulgaricus* 210, *Lactobacillus confusus* ATCC 10881, *Lactobacillus fermentum* 238 and *Escherichia coli* ATCC 23716.

Milk and Cheese Samples:

Raw milk was obtained from the Oregon State University dairy farm whereas pasteurized milk and cottage cheese were purchased from local markets. All samples were kept below 5°C during transportation, inoculation and extended incubation.

Associative Growth Raw Milk:

Raw milk in 100 ml dilution bottles was inoculated with *Lactobacillus* AS-1 at approximately 1 x 10^7 CFU/ml at zero time. The CFU/ml of the natural flora was determined over a one week period of storage at 5°C. *Pseudomonas* isolation agar (Difco) was used to enumerate *Pseudomonas* spp. with incubation at 30°C for 48 hours, whereas other gram negative bacteria were recovered in MacConkey Agar (Difco) using the Spiral Plating System (Spiral System, Inc., Cincinnati, Ohio) with incubation of plates at 37°C for 48 hours.

Effects of the level of *Pseudomonas* spp.:

Raw milk was artificially inoculated with different levels (10^2-10^5 CFU/ml) of *Pseudomonas* spp. mentioned above. The CFU/ml of

Lactobacillus AS-1 in raw milk was 10^7 CFU/ml at the beginning of cold storage for a week. *Pseudomonas* spp. as well as other gram negative bacteria were enumerated during refrigeration using the selective media mentioned above.

Degree of Inhibition by AS-1 Strain:

Lactobacillus AS-1 was added to raw milk at 10^2-10^7 CFU/ml at zero time. Control and inoculated raw milk were kept at 5°C for one week during which time the level of gram negative bacteria was determined.

Pasteurized Milk Experiment:

Pasteurized milk (10^2 CFU/ml) was inoculated with 10^6-10^7 CFU/ml of *Lactobacillus* AS-1 and stored at 5°C for seven days. *Pseudomonas* spp. were monitored during the cold storage period using *Pseudomonas* Isolation Agar. AS-1 was activated before inoculating milk in 11% NDM rather than MRS broth since the latter caused a slight flavor change when added to milk and cottage cheese.

Cottage Cheese Study:

Dry curd cottage cheese (200 g) was creamed with 60 ml of half and half (12% milk fat). Cheese was inoculated with approximately 1 x 10^3 of either *Pseudomonas putida* W or *P. nigrificans.* Meanwhile, active cultures of *Lactobacillus* AS-1 were harvested from MRS broth (30°C for 18 hours) by centrifugation and resuspended to give 10^7 CFU/g and added to creamed cheese. Incubation was at 5°C for 4 weeks during which taste, pH and appearance of cheese were recorded.

Mechanism of Inhibition by *Lactobacillus* AS-1:

a. Hydrogen Peroxide (H_2O_2): The production of H_2O_2 in raw milk inoculated with *Lactobacillus* AS-1 was assessed during 5°C incubation for seven days. Perid Test Strips, purchased from

Boehringer-Mannheim , Germany was used for this purpose. To remove any undetectable residual H_2O_2, Catalase (Sigma) was added at 1% level to raw milk inoculated with *Lactobacillus* AS-1 and incubation was carried out at 5°C for seven days. Raw milk with only AS-1 but no catalase was used as a control. Selective media for *Pseudomonas* spp. and enteric gram negative bacteria were used

b. Lactic Acid Assay and Difference On Growth: Lactic acid (DL-Lactic acid) produced by *Lactobacillus* AS-1 in raw milk at 5°C was assayed using the enzymatic kit of Boehringer Company (catalogue number 139084 and 736-970). To test the influence of lactate on growth, control raw milk (without AS-1) was supplemented with DL-lactic acid (10% sterile solution) to correspond to the level of acid produced by strain AS-1. Eight hours after this acid addition to control raw milk held at 5°C, *Pseudomonas* Isolation Agar and MacConkey Agar were employed to enumerate gram negative bacteria in both test samples and control. The same procedure was repeated after 3, 5, and 7 days of cold storage of raw milk with or without (control) *Lactobacillus* AS-1.

c. Carbon Dioxide (CO_2) Determinations: Concentrations of CO_2 produced by AS-1 were calculated using the enzymatic kit purchased from Sigma (Catalogue # 132-A) along with standard solutions of CO_2 (Sigma, 955-11). The level of CO_2 was determined in raw milk plus AS-1 after 2, 4, 7 and 14 days at 5°C. Also, the level of CO_2 in control raw milk was assessed similarly.

Homo- vs. Heterofermentative Lactobacilli:

Amount of inhibition of various gram negative bacteria in raw milk was compared using *Lactobacillus bulgaricus* 210 (homofermentative) as well as *Lactobacillus fermentum* 238 and *Lactobacillus confusus* ATCC 10881 (heterofermentative) in addition to *Lactobacillus* AS-1. Cultures of the aformentioned bacteria were

activated in MRS broth at 30°C for 18 hours and then raw milk (at 5°C) was inoculated (3% inoculum) with strain AS-1, 238, 10881, or 210. Control raw milk without lactic acid cultures was used as well. Levels of *Pseudomonas* spp. and other gram negative bacteria were determined after 4 and 7 days of cold storage of raw milk (both with and without any of the lactobacilli strains).

RESULTS AND DISCUSSION

Antagonistic Activity of *Lactobacillus* AS-1:

As seen in Figures 2.1 and 2.2, adding AS-1 to raw milk suppressed the growth of gram negative bacteria and maitained them at 10^2 CFU/ml over a 6 day period. However, in the controls viable counts increased from 10^2 to 10^6 CFU/ml over the same time period. Contaminants reaching a level of 10^6 CFU/ml or more is deterimental to the quality of dairy products made from such raw milk due to the production of proteases and lipases by *Pseudomonas* spp. Also, *Lactobacillus* AS-1 was found inhibitory to some food pathogens, especially gram negative bacteria (3). Other mesophilic LAB also have been reported to be antagonistic against spoilage organisms in milk (8, 9, 19, 21, 28, 30, 33). However, inhibition of gram negative bacteria was not acheived and lowering the pH by viable LAB below 6 was required for inhibition (8, 19, 21, 28). However, in the present study inhibition of gram negative bacteria by AS-1 strain was acheived when pH of raw milk was above 6.2. Since heating milk with pH values below 6 may cause clogging of the heat exchanger during pasteurization,the relative high pH in this case is noteworthy. In fact, raw milk inoculated with AS-1 (at 10^7 CFU/ml) followed by incubation at 5°C for six days and then pasteurized (63°C for 35 minutes) did not reveal any abnormal clotting or flavor defects. Other researchers have reported the ineffectiveness of some LAB in restraining the growth of spoilage organisms (19, 33), emphasizing the importance of variations between strains in ability to inhibit undesirable bacteria with which they may be present.

Initial Microbial Load of Raw Milk:

When raw milk was initially inoculated with $\leq 1 \times 10^3$ CFU/ml of *Pseudomonas putida*, *Lactobacillus* AS-1 was able to maintain their number below that level by completely inhibiting their proliferation over a six-day period at 5°C (Figure 2.3). Furthermore, the CFU/ml of *Pseudomonas* spp. decreased by approximately one log, to less

than the initial level (10^5 CFU/ml) present. It is unlikely that raw milk would have more than 10^5 CFU/ml as natural microflora though AS-1 was able to maintain their numbers below this level.

Variations in Antagonistic Activity:

Figure 2.4 shows that a level of 1 x 10^7 CFU/ml of strain AS-1 added initially to refrigerated raw milk maintained the load of gram negative bacteria at 10^2 CFU/ml for 6 days and perhaps longer However, *Lactobacillus* AS-1 at 10^3 or 10^5 CFU/ml provided only slight inhibition in comparison with the control, especially before 3 days of incubation. Over 6 days of refrigeration, only 10^7 CFU/ml of AS-1 was able to maintain the number of gram negative bacteria at 10^2. Thus a dose effect is clear, suggesting some minimum concentration of AS-1 cells or metabolite(s) is necessary to prevent outgrowth of the psychrotrophs. Levels of gram negative bacteria were counted using Pseudomonas Isolation Agar (for *Pseudomonas* spp.) and MacConkey agar (for enterics) at 30°C and 37°C, respectively using the Spiral Plating System. Levels of AS-1 less than 1 x 10^6 CFU/ml were not able to inhibit the growth of contaminants and their counts were not significantly different from controls (Figure 2.4). In this regard, levels of LAB added to raw milk below 10^7 were reported earlier to be ineffective in inhibiting *Pseudomonas* spp. (9, 19). Our strain at this level (1 x 10^7 CFU/ml) was successful in inhibiting food pathogens, in particular gram negative bacteria (3).

Pasteurized Milk:

Figure 2.5 illustrates the strong antagonistic activity of *Lactobacillus* AS-1 also on the natural microflora of pasteurized milk. The CFU/ml of control pasteurized milk increased more than 1,000 folds over one week at 5°C. Post-processing contamination of pasteurized milk in conjunction with temperature abuse (>5°C) would certainly effect the keeping quality and flavor of milk. LAB such as *Lactobacillus* AS-1 maintained the bacterial load (*Pseudomonas* spp.) of pasteurized milk (Figure 2.5) below 10^3 CFU/ml. There have been no reports on extending the shelf-life of pasteurized milk using LAB. The question

remaining to be answered is the legality of adding live cells of LAB to pasteurized milk during refrigeration in order to counteract post-processing contamination. Adding AS-1 to pasteurized milk during refrigeration (5°C) slightly reduced the pH but with no dramatic change in flavor.

Effect of Adding *Lactobacillus* AS-1 to Cottage Cheese:

Cottage cheese artificially contaminated with *Pseudomonas* spp. started to show signs of spoilage after 10 days at 5°C. Slight yellowish appearance as well as abnormal flavor (such as soapy taste) were found. However, cottage cheese protected by *Lactobacillus* AS-1 maintained normal characteristics in terms of flavor and appearance, with a slight sourness. pH of control cottage cheese was 5.7 whereas that of protected cheese was 4.9. Growth of *Pseudomonas* spp. was retarded by *Lactobacillus* AS-1 which in turn extended the shelf-life of the cheese. Methods to extend the shelf-life of food have been mentioned in the literature. Microgard™ (1), a metabolite of *Propionibacterium shermanii,* proved to be a potent inhibitor of gram negative bacteria in cottage cheese and other acid type foods (29). Other approaches, including use of diacetyl-producing LAB (13), CO_2 (10,11,15), and sorbic acid (32) have been used to restrain the growth of undesirable bacteria in this product. Nisin, a polypeptide produced by certain strains of *Lactococcus lactis* was found inhibitory to the pathogen, *Listeria monocytogenes* in cottage cheese (5).

Hydrogen Peroxide (H_2O_2):

When AS-1 was grown in raw milk, no H_2O_2 was detected (Perid test) even after 8 days at 5°C. Furthermore, adding catalase did not abolish the antagonistic activity of AS-1 towards gram negative bacterial pathogens in raw milk (3). This suggests that H_2O_2 is not responsible for the inhibitory activity of AS-1. Furthermore, the inhibition of gram negative bacteria (*Pseudomonas* spp.) in pasteurized milk as mentioned above supports the idea that H_2O_2 is not a factor in the antagonistic activity of AS-1. H_2O_2 is known to

activate the lactoperoxidase system of raw milk but this system is destroyed by pasteurization. The involvement of H_2O_2 in inhibiting undesirable organisms has been reported in raw milk inoculated with LAB (16, 21, 28).

Role of Lactic Acid:

Amounts of lactic acid (D and L isomers) produced by *Lactobacillus* AS-1 are seen in Figure 2.6. Interestingly, DL-lactic acid added artificially to control raw milk (without AS-1) to get to the same pH as that of raw milk-containing AS-1 did not antagonize the growth of *Pseudomonas* spp. or other gram negative bacteria (Figure 2.6). No inhibition was exerted by DL-lactic acid added even when the pH of control raw milk was adjusted to 6. The same results were obtained when sodium acetate was added to acheive the same pH which strain AS-1 produced in raw milk , indicating that acetate at pH 6 has a minimum inhibitory activity in refrigerated raw milk at this pH. It is well known that acetic acid at a pH near or below its pKa (4.8) is inhibitory to many microorganisms. However, at pH 6.0 about 93% of this acid is ionized, in which form it can not penetrate bacterial cells. However, *Lactobacillus* AS-1 in raw milk was able to maintain the number of gram negative bacteria at 10^2 even when the pH was as high as 6.4 after 3 days at 5°C (Figure 2.6). This suggests that lactic acid was not a major factor in inhibiting gram negative bacteria, especially at pH values above its pKa (3.8). There have been conflicting reports on the role of lactic acid in antagoniziing *Pseudomonas* spp. in raw milk. While Ross, (30) and Champagne, (8) excluded the role of lactic acid produced by LAB in inhibiting *Pseudomonas* spp., Champagne et al., (9), Griffiths et al., (19) and Reinheimer et al., (28) emphasized the major role of lactic acid in inhibition.

Carbon Dioxide Content:

The enzymatic method indicated that the concentrations of CO_2 in the cold (5°C) raw milk inoculated with *Lactobacillus* AS-1 were 2.2, 4.6, 5.0 , and 28 mM after 2, 4, 7, and 14 days, respectively. No CO_2 was

detected in control raw milk without the AS-1 strain. Higher amounts of CO_2 were produced by AS-1 in MRS broth (28 mM) in comparison with 11% NDM (4mM) after incubation at 30°C for 23 hours. pH of raw milk inoculated with AS-1 strain for 6 days went up (0.2 units) by vigorous stirring at room temperature as a result of CO_2 eliminiation by agitation. CO_2 addition to milk and meat has been reported to inhibit *Pseudomonas* spp. and other spoilage organisms (10,15,23, 27,29). However, higher concentrations (>10 mM) were used in some of those studies. The inhibitory activity of CO_2 may be enhanced by lowering the storage temperature since CO_2 is more soluble at lower temperatures. The mechanism by which CO_2 inhibits bacterial growth is still unknown, though some believe that it disrupts cell permeability and enzymatic activity (22). However, lowering the pH and decreasing dissolved oxygen upon addition of CO_2 was reported by Roberts and Torrey (29) to be unrelated to the bacteriostatic affect of CO_2. It is clear that more research is needed to prove or disprove the presumed inhibitory effect of CO_2 produced by *Lactobacillus* AS-1 in refrigerated milk.

Comparisons Among Different Species of Lactobacilli:

As seen in Figure 2.7, the growth of *Pseudomonas* spp. and other gram negative bacteria was dramatically restricted in raw milk only in the presence of *Lactobacillus* AS-1 when holding the milk for 7 days. Since *Pseudomonas* are psychrotrophic organisms, they increased in numbers at 5°C over the one week period by more than 10,000 folds (10^2 to $>10^7$ CFU/ml) in the presence of other *Lactobacillus* species. Other works also have observed variability between different cultures in causing inhibition of *Pseudomonas* spp. (21, 33). This again emphasizes the unique ability of AS-1 strain to inhibit gram negative bacteria also present in milk. Especially noteworthy is that a known heterofermentative *Lactobacillus*, *L. fermentum*, which like AS-1 produces CO_2 from glucose derived from lactose, shows no inhibitory activity. Therefore, either *L. fermentum* produces significantly less CO_2 than AS-1 or AS-1 inhibits organisms by a mechanism other than CO_2 production. In a previous study (3), *Lactobacillus* AS-1 was found to be a stronger inhibitor of gram

negative, food-borne bacterial pathogens when compared with lactococci, lactobacilli, *Leuconostoc*, *Propionibacterium* and pediococci.

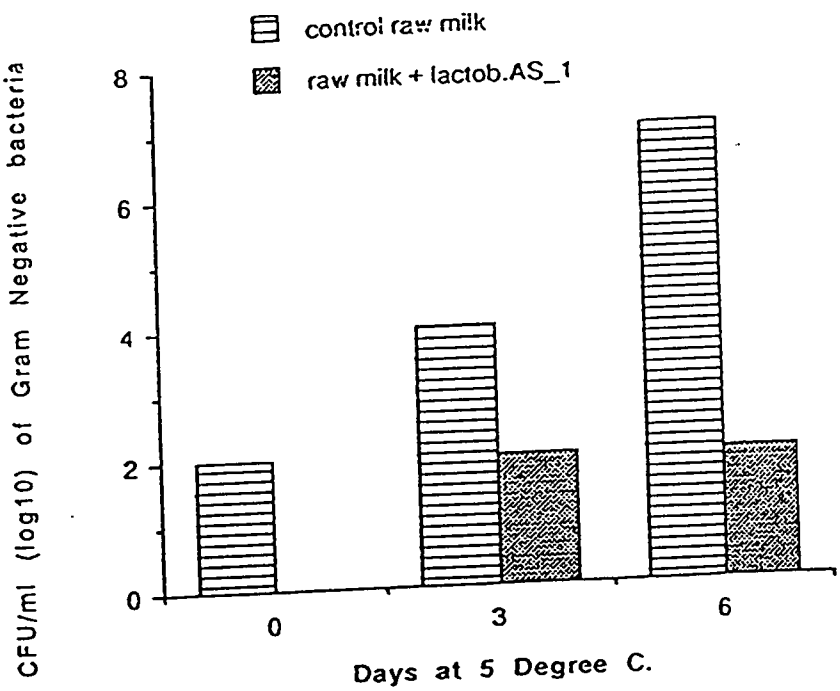

Figure 2.1: Effect of addition of 10^7 CFU/ml of *Lactobacillus* AS-1 to refrigerated (5°C) raw milk on growth of the natural gram negative bacterial flora of the milk when held for six days. Pseudomonas Isolation Agar and MacConkey Agar were used to recover gram negative bacteria at 30°C and 37°C, respectively, for 48hours.

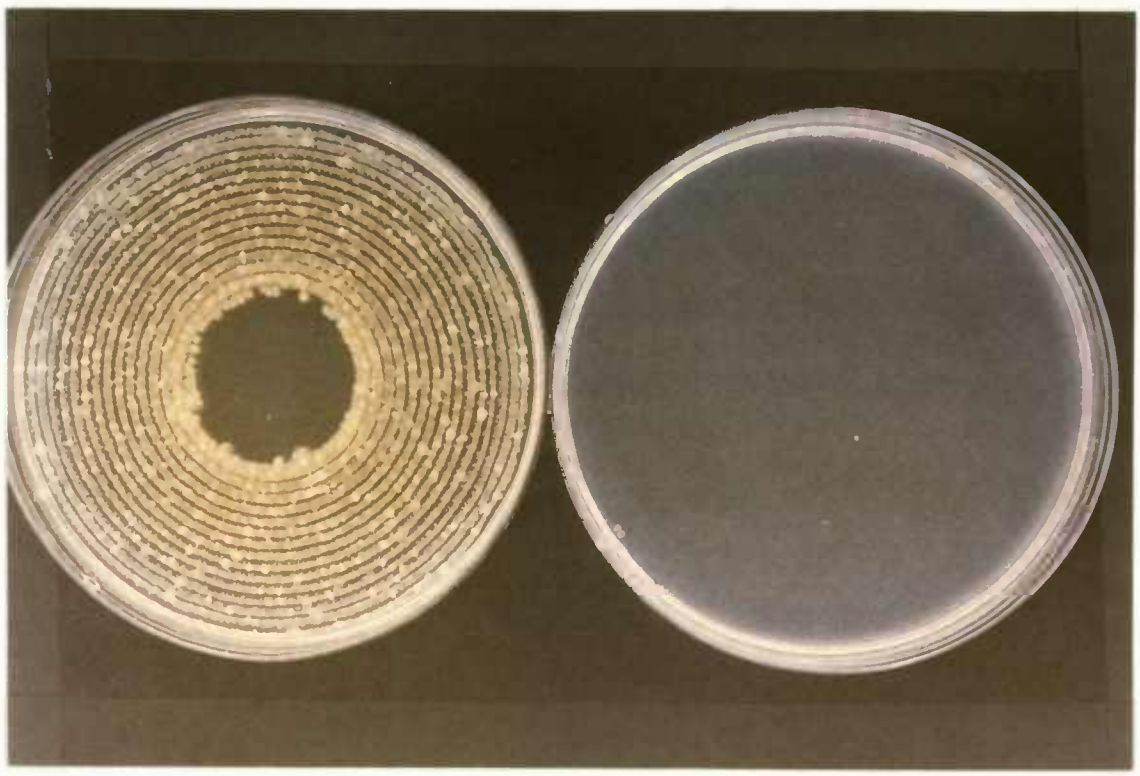

Figure 2.2: Growth of *Pseudomonas* spp. in raw milk ($>10^7$CFU/ml) in the abscence of *Lactobacillus* AS-1 for 7 days at 5°C (left). Inhibition of these bacteria ($<10^2$CFU/ml) was acheived by AS-1 under the same conditions (right). Pseudomonas Isolation Agar was the selective medium with incubation at 30°C for 48 hours.

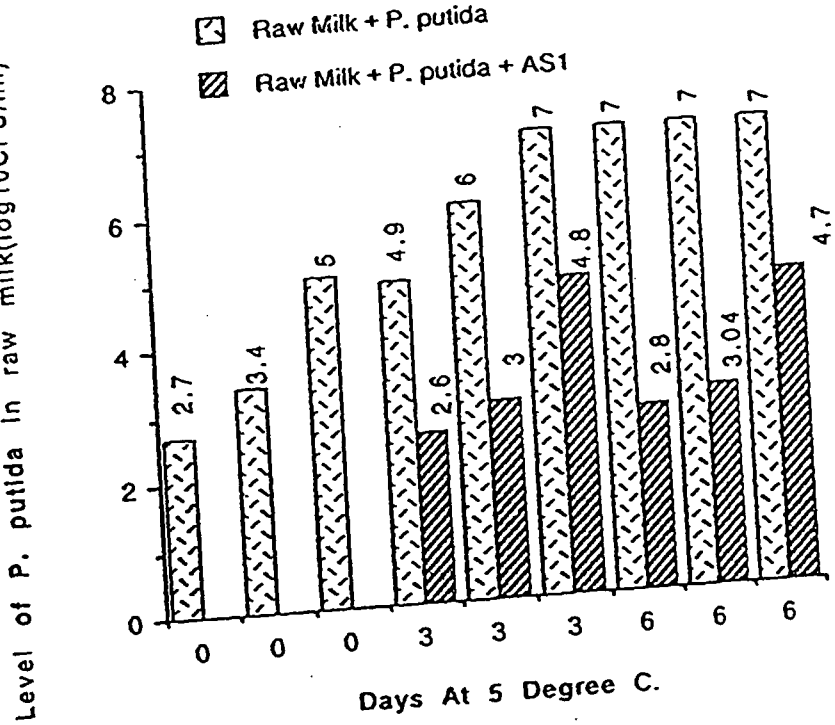

Figure 2.3: Influence of initial CFU/ml of *P. putida* added to raw milk on inhibition by *Lactobacillus* AS-1 over 6 days at 5°C. Pseudomonas Isolation agar was the selective medium.

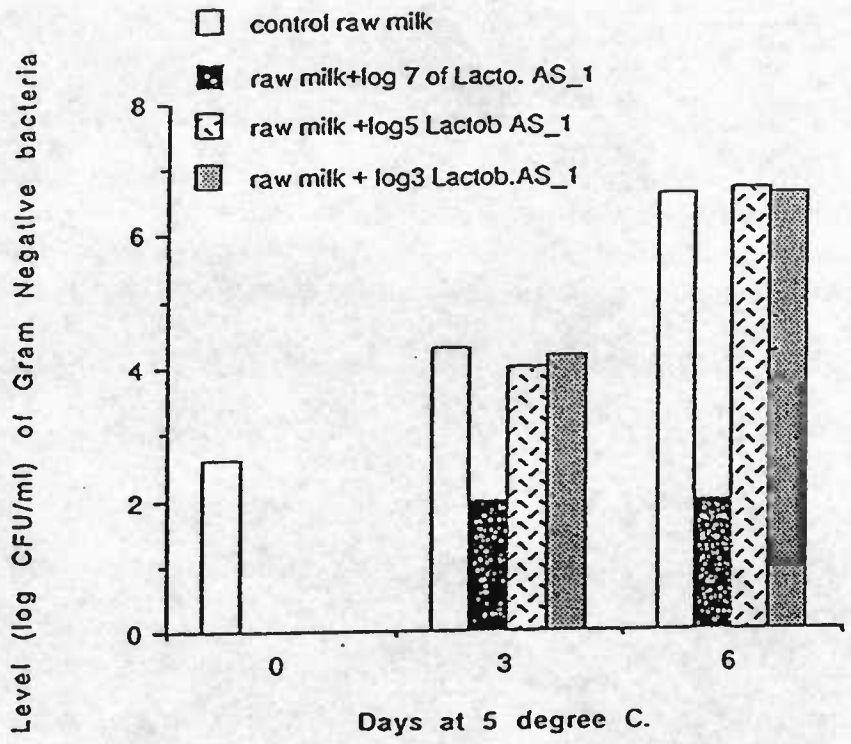

Figure 2.4: Effects of different levels of *lactobacillus* As-1 on the growth of natural, gram negative bacterial flora of raw milk held at 5°C for six days. initial CFU/ml of natural microflora was 4 X 10^2.

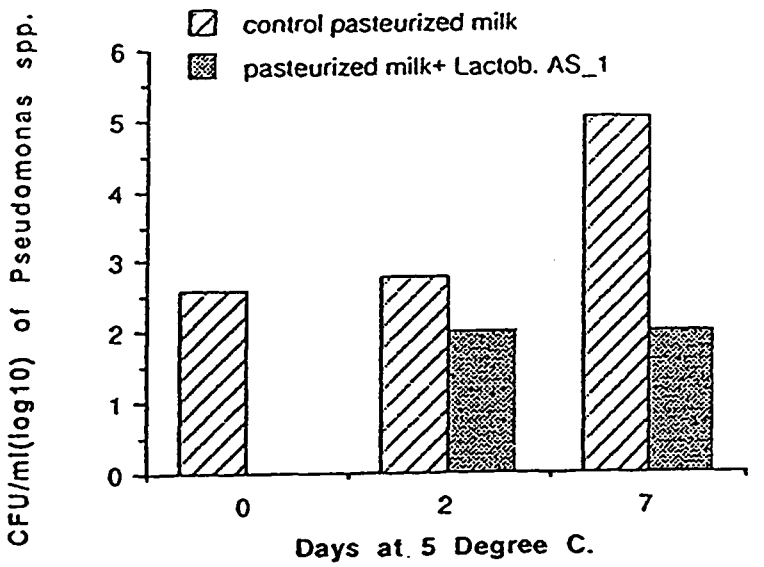

Figure 2.5: Counts of natural microflora of pasteurized milk held at 5°C for 7 days without (control) and with 10^6-10^7 CFU/ml *Lactobacillus* AS-1.

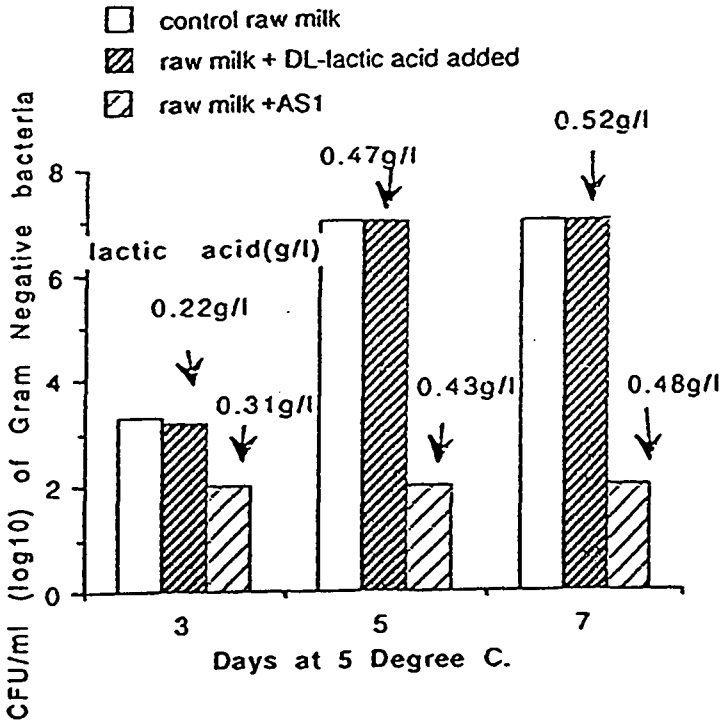

Figure 2.6: Effect of DL-lactic acid produced by *Lactobacillus* AS-1 or artificially added to adjust the pH of raw milk on inhibiting the growth of the natural gram negative bacteria . pH of raw milk plus As-1 was 6.4, 6.3, and 6.1 after 3, 5, and 7 days, respectively at 5°C.

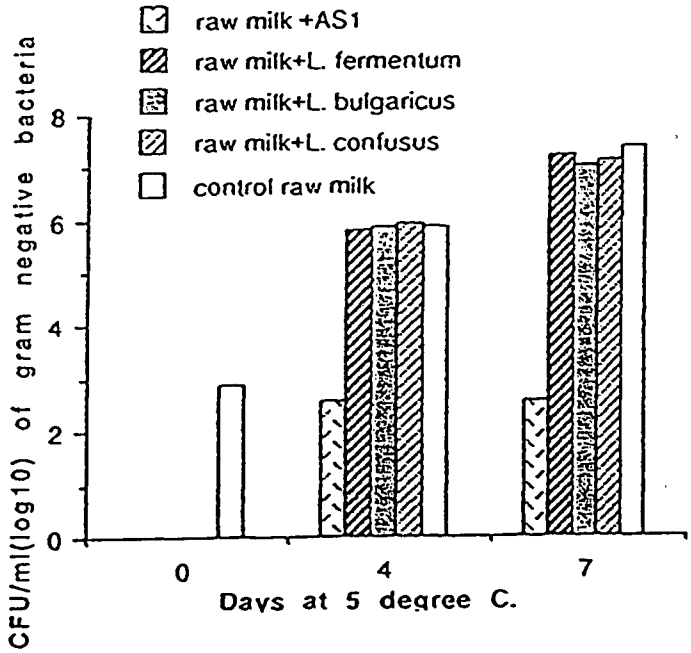

Figure 2.7: Effect of the presence of four different *lactobacillus* species added (10^7CFU/ml) to refrigerated raw milk on the growth of the natural raw milk flora over 7-day holding period.

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CHAPTER 3

Highly Selective Medium for Isolation of *Listeria monocytogenes* from Food

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ABSTRACT

A new selective medium, Al-Zoreky-Sandine listeria medium (ASLM), was formulated to recover *Listeria monocytogenes* from food specimens; the medium completely inhibited common food microflora. Recognition of *Listeria* colonies is evident by black discoloration of the medium due to esculin hydrolysis without need for special illuminating equipment. The medium contains acriflavin, ceftazidime, and moxalactam as selective agents. Compared with Listeria Selective Agar, ASLM was equally effective in recovering *Listeria monocytogenes* However, ASLM inhibited micrococci, enterococci, and gram-negative bacteria, especially a strain that mimicked *Listeria monocytogenes* on Listeria Selective Agar. The new medium was able to recover heat injured cells with only 15% less count than the nonselective medium.

INTRODUCTION

Recent food outbreaks of listeriosis (6, 9, 12, 15, 20) have emphasized the need for a highly selective medium to detect *Listeria monocytogenes*, a human and animal pathogen. Listeriosis has a mortality rate of up to 33% (5, 17) and is highest for pregnant women, infants, and immunocompromised persons. Fifty percent lethal doses of *Listeria monocytogenes* are as low as 5 cells for immunocompromised mice whereas the 50% lethal dose for normal mice is 10^5 to 10^7 cells (7). The natural flora of food may complicate the detection of the pathogen with the media now available. Antibiotics and chemicals are being used in most media in an attempt to recover listeriae while inhibiting other flora (1, 5, 6, 8). However, interference from background microorganisms, difficulty in recognition of listeriae, and the need for special lighting for identification have emphasized the need for research on *Listeria* isolation media (3, 10, 11, 13, 24). A monoclonal antibody-based-enzyme-linked immunosorbent assay has been described, but the test requires the presence of high levels of listeriae ($>10^4$ CFU/ml) in foods for positive detection (2, 19, 21). It was the objective of this study to evaluate a newly developed medium Al-Zoreky-Sandine listeria medium (ASLM), for isolation of *Listeria monocytogenes* from foods, especially to determine the amount of interference from naturally present bacteria.

MATERIALS AND METHODS

Cultures and strain maintenance:

Listeria monocytogenes Scott A, Jalisco, and ATCC 7644 and *Listeria innocua* were used after being activated in brain heart infusion (BHI) broth (Difco Laboratories) at 37°C for 18 to 24 h. All strains were maintained between transfers on tryptic soy agar (TSA) slants at 2 to 5°C.

Test Media:

The experimental medium (ASLM) consisted of Listeria Selective Agar (LSA) Base (CM856; Oxoid Ltd.), which contains the following (per liter): Columbia blood agar base, 39 g; esculin, 1 g; ferric ammonium citrate, 0.5 g; and lithium chloride, 15 g. After the base medium was autoclaved at 121°C for 15 min and tempered to 45°C in a water bath, the following inhibitory agents (filter sterilized, 0.2 μm-pore-size filter) were aseptically added to 1 liter; 5 ml of aqueous absolute ethanol (1:1) containing 200 mg of cycloheximide (Sigma Chemical Co.), 2.5 ml of an aqueous solution containing 50 mg of ceftazidime pentahydrate (Glaxo), 2.5 ml of an aqueous solution containing 10 mg of acriflavin hydrochloride, and 2 ml of a 1% solution of moxalactam (Sigma) in phosphate buffer (pH 6.1). The final pH was 7 ± 0.1.

The other medium used for comparative purposes was LSA, the so-called Oxoid agar, with an antibiotic supplement (Oxoid); this medium gave the highest recovery of *Listeria monocytogenes* in comparison with other well known listeria media (20). TSA was used as the reference agar (nonselective).

Spiral Plating:

Quantitative evaluation of recovered *Listeria monocytogenes* was carried out with a Spiral Plater (model D; Spiral Systems) unless

otherwise mentioned. Active cultures were diluted to 10^4 to 10^5 CFU/ml. Incubationwas at 37°C for 48 h, and the number of cells was determined with a counting grid.

Pure culture comparison:

Each *Listeria monocytogenes* (Scott A, Jalisco, or ATCC 7644) or *L. innocua* strain was activated and surface plated (Spiral Plater). Both ASLM and Listeria Selective Agar (LSA) were used to enumerate the pathogen. The nonselective medium TSA was the control. Statistical analysis was conducted to evaluate any significant differences between the media by using analysis of variance (Statgraphic version 4.0, Oregon State University).

Selectivity of ASLM.

Several microorganisms (Table 3.1), including *Listeria monocytogenes* strains as positive controls, were activated in BHI broth for approximately 24 h at 37°C. Samples (0.1 ml) of the cultures were spread onto the surface of TSA, ASLM, and LSA with a glass rod. Plates were incubated at 37°C for 48 h. Plates were examined for microbial growth. For lactic acid bacteria, MRS agar was used as a reference medium, and KF streptococcal medium was used as a control medium for *Streptococcus faecalis*.

Recovery of *Listeria monocytogenes* from food.

Raw milk obtained from the Oregon State University dairy farm was artificially contaminated with *Listeria monocytogenes* (ATCC 7644 or Scott A) at 10^4 to 10^5 CFU/ml. Cells were plated on ASLM and LSA (Spiral Plater). In addition, the level of natural microflora was determined by using TSA. Plates were incubated at 37°C for 48 h. Similarly, pasteurized milk (2% fat) was contaminated at 10^4 to 10^5 CFU/ml. Also, samples of raw and pasteurized milk not contaminated with *Listeria monocytogenes* were plated in the above-mentioned media as a control. In other experiments, samples of raw and pasteurized milk were incubated at 20°C for 18 h or at 2 to 5°C for 10

days to enhance growth of the natural flora before being artificially contaminated with *Listeria monocytogenes*. Cottage cheese was also inoculated with *Listeria monocytogenes* at approximately 10^4 CFU/ml (ATCC 7644) or 10^5 CFU/ml (Scott A), and samples were suface plated on TSA, ASLM and LSA with a glass rod. Uninoculated control Cottage cheese was plated as well.

Heat-injured cells:

The procedure of Golden et al. (13) was employed with *Listeria monocytogenes* ATCC 7644 as the test organism. Cells ($\approx 10^8$ CFU/ml) were heated (52°C for 15 min.) in a water bath in tryptose-phosphate broth (TPB). After heating and cooling the organism was diluted in TPB to give approximately 10^2 to 10^6 CFU/ml. The Spiral Plater was used, and plates were incubated at 37°C for 48 h. Both heat-injured and noninjured cells (controls) were plated in ASLM. The control medium used was TSA.

RESULTS AND DISCUSSION

Colonial appearance of *Listeria monocytogenes* and productivity on ASLM:

L. monocytogenes (Scott A, Jalisco, and ATCC 7644) or *L. innocua* grew on both LSA and ASLM as black colonies surrounded by black, which was due to esculine hydrolysis (8, 12). Inspection of listeriae on ASLM further revealed that they had small dark centers; the rest of the colony appeared dark green. Also, these colonies exhibited sunken centers. No significant difference ($P>0.01$) was found between the number of listeria colonies appearing on either medium (Table 3.2). Another independent study (data not shown) with both media also indicated that ASLM and LSA gave the same recovery of *L. monocytogenes* (M. A. Daeschel [Department of Food Science and Technology, Oregon State University, Corvallis], personal communication). Also, ASLM was not inhibitory to the three *L. monocytogenes* strains used when compared with the non-selective medium. Other media have been found inhibitory to *Listeria* spp. (13, 18, 24) and to provide poor colony recognition (3).

Selectivity of the test medium:

ASLM was strongly inhibitory (100% inhibition) for all nonlisterial microorganisms tested (Table 3.1). Both strains of *L. monocytogenes* grew in LSA and ASLM used as positive controls. In fact, both staphylococci and *S. faecalis* GF590 and CG110 grew well on LSA and gave positive esculin reactions, indicated by the black discoloration of the medium; also, one esculin-negative staphylococcus grew luxuriously on LSA but was inhibited on ASLM. The black color on LSA medium can be misleading for identification of listeriae, since staphylococci and enterococci may be present in food or clinical speciments and give false-positive readings on LSA. Most important is that complete inhibition of all organisms tested was achieved in ASLM, even though high number of cells were spread onto the surface. With prolonged incubation (>48 h), *S. faecalis* GF590 grew

very slightly in ASLM to produce pinpoint colonies, whereas a complete black discoloration in LSA plates occured in less than 24 hr of incubation. Furthermore, strains of aerobic spore formers and diphtheroids caused discoloration in LSA (8). Other media suggested for listeriae also have been found unable to suppress *Enterococcus* spp and micrococci (1, 16, 18, 24). In addition, the use of Henry illumination to view *Listeria* colonies on LPM and MMA media (11) can be misleading, since some members of a natural flor can give the same blue color as that produced by *Listeria* spp. (5, 16). The combination of inhibitory agents used in ASLM (moxalactam, ceftazidime, and acriflavin) proved superior in inhibiting a variety of microorganisms while allowing listeriae to grow. For more details concerning the inhibitory substances used in different listerial media, refer to Cassiday and Brackett (5).

Recovery of *L. monocytogenes* from food:

Results for recovery of *L. monocytogenes* from food are summarized in Table 3.3. More cells of ATCC 7644 were recovered on ASLM than on LSA. However, both media gave comparable results with the Scott A strain. Other selective media have been reported to have different recovery rates for isolation of *L. monocytogenes* from various foods and clinical specimens (8, 10, 13, 20). It is noteworthy that recovery of *L. monocytogenes* from food with ASLM was efficient, since a high number ($\approx 10^6$ CFU/ml) of background microflora also was present. Incubation of raw milk at 20°C for 20 h or 2°C to 5°C for 10 days before inoculation with *L. monocytogenes* made detection of the pathogen impossible on LSA, since many staphylococci emerged ($>10^6$ CFU/ml) that were esculin positive (black discoloration of the medium) (Fig. 3.1). Furthermore, a gram-negative bacterium isolated from cabbage that grew slowly at 25°C and not at all at 30°C and that gave an esculin-positive reaction on LSA was completely inhibited on ASLM. In this regard, other recommended media for *L. monocytogenes*, AC and MVJ (1, 4), allowed the growth of other natural contaminants present in raw milk and gave colony characteristics similar to those of *L. monocytogenes* (data not shown). Also, microscopic examination of staphylococci grown in LSA

was misleading, since under these conditions this organism had unusually long cells that tended to be gram negative. By culturing these isolates in BHI broth, cells were recovered as typical gram-positive cocci. Raw milk tested in the present study was free of *L. monocytogenes* even after prolonged cold storage. This pathogen, however, has been recovered from raw milk at levels of 1.5% and about 7% in Canada and the United States, respectively, and as high as 45% in Spain (9, 22). The high number of natural contaminants encouraged by preincubation in our study did not complicate the detection and identification of *L. monocytogenes* in ASLM, although the interference of contaminating organisms has been noted with other media (1, 3, 6, 8, 10, 18).

Recovery of heat-injured cells:

Heat injury of LM ATCC 7644 caused approximately 15% lower colony count in ASLM when compared with that of the nonselective medium TSA (Table 3.4). An attempt to resuscitate cells after heat injury and before plating may increase the number of cells recovered. It has been stated that a 1,000 times lower count was achieved when *L. monocytogenes* was heat injured at 52°C for 60 min and then plated on a medium containing sodium chloride (14, 23). It also has been noted that heat injury of this pathogen caused a 20 to 95% decrease in the count, depending on the strain and medium, in comparison with the count on nonselective medium (16). Similarly, other media have been found to give poor recovery of injured listeria cells (13, 24).

Table 3.1: Selectivity of ASLM for listeriae.

Microorganism	Growth in[a]: TSA	ASLM	LSA
Gram-negative bacteria			
Pseudomonas aeruginosa ATCC 419	+++	-	-
Escherichia coli V517	+++	-	-
Salmonella typhimurium	+++	-	-
Yersinia enterocolitica ATCC 23715	+++	-	-
Aeromonas hydrophila ATCC 7965	+++	-	-
Campylobacter jejuni ATCC 29428[b]	+++	-	-
Gram-positive bacteria			
L. monocytogenes ATCC 7644	+++	+++	+++
L. monocytogenes Scott A	+++	+++	+++
Lactobacillus bulgaricus CH_2	NT	-	-
Lactobacillus lactis LAR 28	NT	-	-
Staphylococcus aureus[c]	+++	-	+++
Staphylococcus isolate[d] (raw milk)	+++	-	+++
Lactococcus lactis ATCC 7692	NT	-	-
Lactococcus diacetylactis 18-16	NT	-	-
L. diacetylactis 26-2	NT	-	-
Streptococcus faecalis GF590	NT	+	+++
S. faecalis CG110	NT	-	+++
Streptococcus thermophilus CR5	NT	-	-

[a]Samples of 0.1 ml of 24-h cultures were spread onto the surface of the agar: +++ = heavy growth; - = no growth; + = very slight growth after 48 h of incubation; NT = not tested (see Materials and Methods).

[b]Grown at 37°C in Campy Pak (BBL Microbiology Systems).

[c]Esculin negative.

[d]Esculin positive.

Table 3.2: Comparison of recovery of *L. monocytogenes* and *L. innocua* on TSA, ASLM, and LSA media.

Strain	Recovery[a] (log_{10} CFU/ml) in:		
	TSA	ASLM	LSA
L. monocytogenes			
Scott A	5.01	5.04	4.99
ATCC 7644	5.19	4.98	5.0
Jalisco	5.15	5.15	5.10
L. innocua	4.63	4.5	4.63

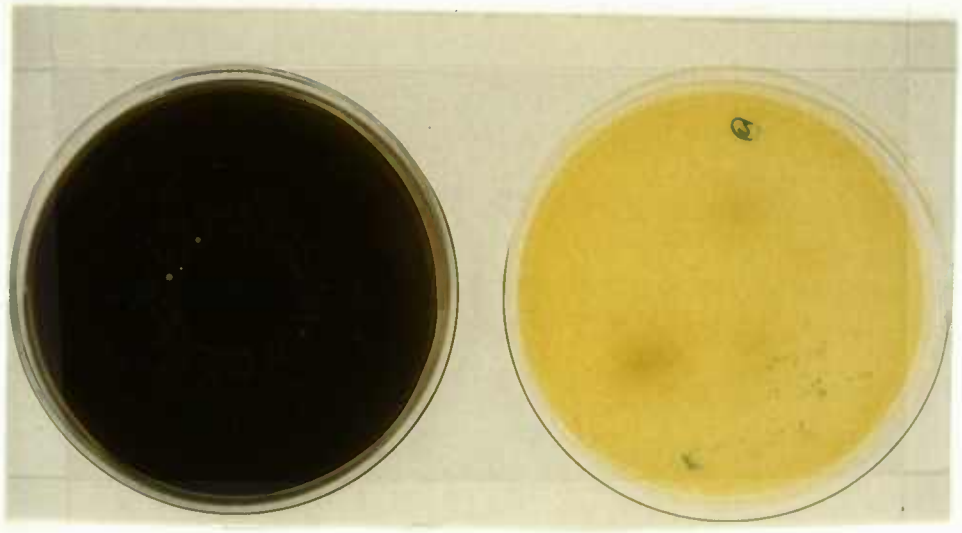
[a]Spiral plate counting with incubation at 37°C for 48 h. Each value represents the average of two replicates in duplicate.

Table 3.3: Efficacy of direct plating on ASLM for recovering *L. monocytogenes* Scott A and ATCC 7644 from food[a].

Strain	Recovery medium	Log10 CFU at indicated inoculum per ml or g of:		
		Raw milk	Pasteurized milk[b]	Cottage cheese
ATCC 7644	ASLM	4.30	5.07	4.12
	LSA	4.00	5.01	4.03
Scott A	ASLM	4.46	5.05	4.3
	LSA	4.49	5.17	4.38

[a]Food was contaminated artificially with 10^4 to 10^5 CFU of *L. monocytogenes* per ml. Control samples (without added listeriae) contained between 10^4 and more than 10^6 CFU of natural flora per ml.

[b]Pasteurized milk was incubated at 20°C for 20 h before being inoculated with *L. monocytogenes*.



Control
Raw milk incubated
at 20°C for 20 hrs
on LSA

Control
Raw milk incubated
at 20°C for 20 hrs
on ASLM

L. m. Scott A
Raw milk inoculated
with 1 x 10^3 CFU/ml
on LSA

L. m. Scott A
Raw milk inoculated
with 1 x 10^3 CFU/ml
on ASLM

Figure 3.1: Appearance of *L. monocytogenes* Scott A colonies when spread on LSA and ASLM in comparison with control plates of these two media. Raw milk samples were preincubated at 20°C for 20 h (controls) before inoculation with *L. monocytogenes*

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CHAPTER 4

Comparative Recovery and Selectivity of Several Media for *Listeria monocytogenes*

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ABSTRACT

Comparative studies to recover *Listeria monocytogenes* were carried out using 5 different agar media. All media gave similar results in recovering the pathogen, except for MVJ (4) which was inhibitory for the bacterium. Growth of staphylococci, enterococci, *Enterobacteriaceae* and yeast was completely inhibited in ASLM (1) while each of the other media allowed their growth.

INTRODUCTION

Listeria monocytogenes (LM) is a gram positive, non-sporeforming rod. The organism was once believed to be a pathogen only of veterinary concern. However, recent food disease outbreaks (10,11,12) associated with this pathogen since the 1980's have established LM as an emerging food pathogen which can grow at refrigeration temperatures (a psychrotroph). The high risk (30% mortality rate) of consuming foods contaminated with LM emphasizes the need for selective methods for its isolation. Different agar media have been designed (1,2,4,8,16,17). However, some of the aforementioned media are either not sufficiently inhibitory to food background flora or they are inhibitory for LM (1,2,6,11,13,16,17 ,19). The recognition of *Listeria* spp. in some media (1,8,16,17) is indicated by esculin hydrolysis (black discoloration). However, esculin-positive, non-listeriae bacteria were recovered in some of these media. Blood haemolysis (B-haemolysis) was employed to identify LM in other media (7,9). Due to the fact that non-haemolytic LM strains and B-haemolytic *Listeria* spp. other than LM do exist (9,10), this may result in either false negative or false positive identification of LM. In view of the previous observations, this study was conducted to compare five agar media for recovery of LM as well as to determine their selectivity.

MATERIALS AND METHODS

Two LM strains (Scott A and Jalisco) were activated in BHI broth (Difco) at 37°C for 24 hours. They then were diluted in 0.1% peptone water (sterile) to obtain approximately 10^5 CFU/ml and used at this concentration for quantitative studies of several selective media using the Spiral Plating System (Spiral Systems, Inc., Cincinnati, OH). Agar media employed were AC (2), ASLM (1), MVJ (4), OX (Oxoid) (8), and PALCAM (Oxoid) (17).

After inoculating agar plates prepared by the Spiral System™ (Cincinnati, OH), they were incubated at 37°C for 48 hours followed by counting using the designed grid of the Spiral System™. Statistical analysis was done to evaluate any significant differences among media by using analysis of variance (ANOVA). LM colonies in OX, ASLM and PALCAM were black due to esculin hydrolysis whereas in MJV black colonies were the result of potassium tellurite reduction. In AC medium, yellow colonies developed. For selectivity, several microorganisms were activated at 37°C in BHI broth before being spread or streaked onto agar surfaces. Plates were incubated at 37°C for 48 hours.

Meanwhile, raw milk was incubated at 20°C for 20 hours to enumerate the background flora. Then samples were spread onto agar surface of each selective medium followed by incubation at 37°C for 48 hours. For both quantitative recovery of LM and selectivity of agar media, tryptic soy agar (TSA) (Difco) was used as the reference medium.

RESULTS AND DISCUSSION

Results obtained (Table 4.1) showed no significant difference ($P>0.05$) in the count (CFU/ml) of LM strains among the four selective media, AC, ASLM, OX, and PALCAM. The exception was MVJ which was found inhibitory for LM. MVJ also was inhibitory to the target pathogen in previous studies (6,11,17). It was interesting to find that MVJ was completely inhibitory to LM strains in some of the trials. However, in other trials different bacitracin and potassium tellurite lots were used which allowed the growth of LM though with significant inhibition. As a result, MVJ was omitted from further studies on quantitative recovery of LM. The variability of results obtained using MVJ was attributed to differences in lot productions of bacitracin and moxalactam (5). Enumerated colonies of LM were seen within 24 hours of incubation in AC, OX, and ASLM, in addition to the nonselective medium (TSA). In PALCAM and MVJ, colonies developed after 24 hours of incubation. Furthermore, smaller colonies were seen in PALCAM and MVJ after 48 hours of incubation in comparison with other media. On AC medium, LM colonies were yellow and surrounded by clear zones. Despite the comparable recovery rate of LM, AC and OX were the only two media which allowed the growth of all nonlisteriae organisms (Table 4.2). The previous two media were reported ineffective in restraining the growth of common food microflora (1, 2, 11, 13, 14, 15, 17, 18). In contrast, MVJ and PALCAM were not inhibitory to *Staphylococcus aureus* and *Enterococcus* spp (Figure 4.1). The aforementioned bacteria gave typical colony characteristics as LM on those two media (Figure 4.1.) which will lead to false positive identifications of LM. Growth of nonlisteriae organisms have been reported using the previous two media (4, 5, 6, 17, 18, 19). On the other hand, ASLM was successfully inhibitory to nonlisteriae genera (Table 4.2 and Figure 4.1). This particular medium (ASLM) strongly suppressed the growth of background microflora of food containing LM (1, 3). Yeasts recovered from raw milk grew in AC, MVJ and PALCAM, which also complicated the detection of *Listeria* spp. in foods containing fungi (19). In contrast, both ASLM and OX contain cycloheximide, inhibitory

for fungi. Furthermore, gram negative rods which grew in MacConkey agar and Hektone enteric agar and gram positive cocci recovered from raw milk grew heavily in AC and OX and even mimicked LM colony characteristics in both media.

MVJ and PALCAM, on the other hand, allowed only the growth of gram positive coccal bacteria of raw milk. Complete inhibition of raw milk background flora was achieved in ASLM.

In summary, while all media tested (AC, ASLM, OX, and PALCAM) were highly similar in quantitatively recovering pure cultures of LM, ASLM was superior in inhibiting a wide range of microbial genera, some of which are present in food and would cause false positive results with media other than ASLM.

Table 4.1. Recovery of *Listeria monocytogenes* strains in several agar media after incubation at 37°C for 48 hours.

Organisms	CFU/ml (log_{10}) on medium				
	TSA	AC	ASLM	OX	PALCAM
L. monocytogenes					
Scott A	$1.7x10^5$ (5.23)	$1.5x10^5$ (5.18)	$1.5x10^5$ (5.18)	$1.6x10^5$ (5.20)	$1.6x10^5$ (5.20)
Jalisco	$1.2x10^5$ (5.08)	$1.4x10^5$ (5.15)	$1.5x10^5$ (5.18)	$1.4x10^5$ (5.15)	$1.4x10^5$ (5.15)

Table 4.2. Selectivity of *Listeria* media toward several microorganisms.

Organisms	Growth in medium				
	AC	ASLM	MVJ	OX	PALCAM
Gram negative bacterium (raw milk isolate)	++	-	-	++	-
Gram negative bacterium (cabbage isolate)	++	-	-	++	-
Staphylococcus aureus	++	-	+	++	++
Enterococcus faecalis (GF590)	++	-	+	++	+
Enterococcus spp. (raw milk)	++	-	+	++	++

++: Heavy growth.

+: Moderate growth.

-: No growth.

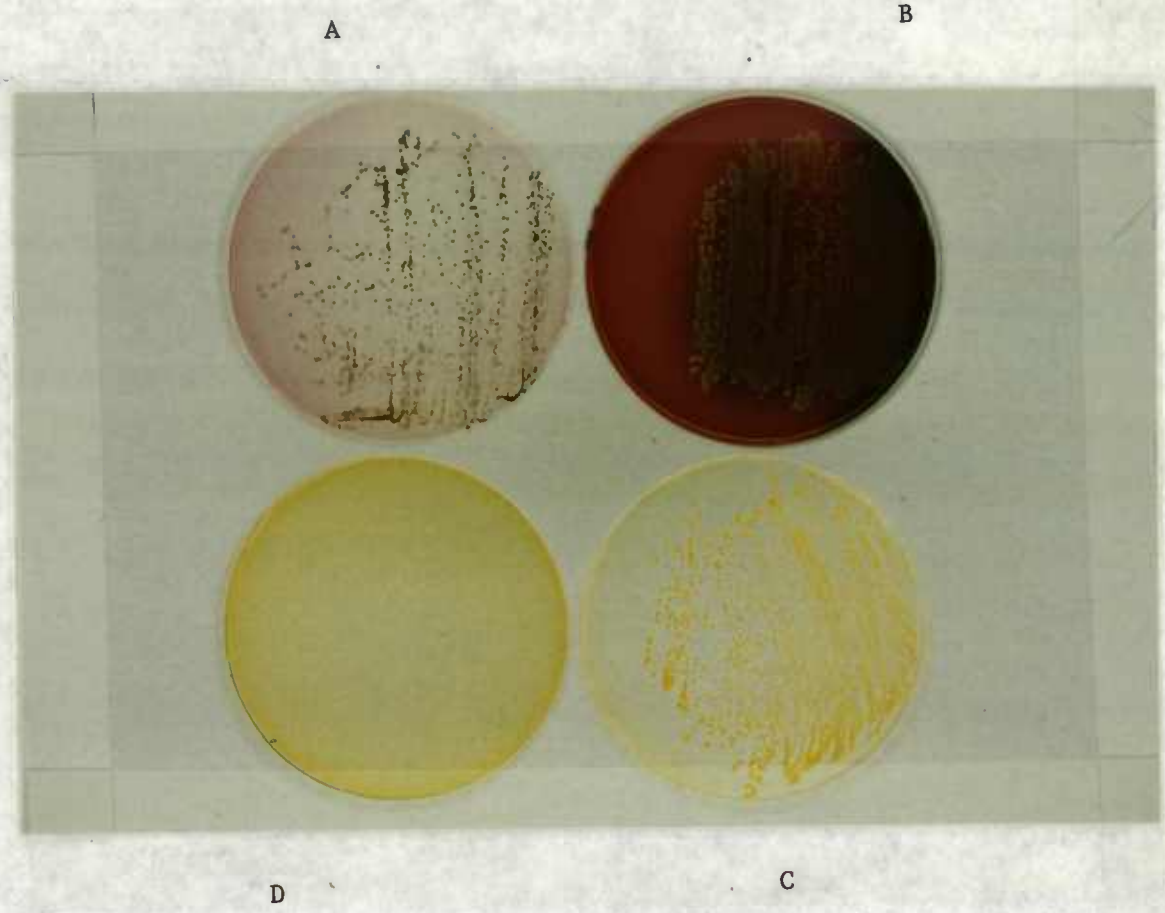

Figure 4.1: Growth of nonlisteriae microorganisms, *S. aureus* and *Enterococcus spp* on listeriae media. Agar media were: MVJ (A) ; PALCAM (B) and AC (C). Both bacteria were completely inhibited on ASLM (D).

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CHAPTER 5

Lactococcus Genus: A selective and Differential Agar Medium

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ABSTRACT

An agar medium (Alsan), was selective for *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*. The former developed white or yellow colonies; the latter gave blue-green or blue colonies. Eleven strains of *L. lactis* subsp. *cremoris* did not grow in this medium. The medium contained phenylethanol agar, lactose, glycine anhydride, lithium chloride and trimethoprim. In comparison with M17 medium, Alsan was not significantly different ($P>0.05$) in recovering either bacterium. Furthermore, the medium inhibited the common food genera *Pseudomonas*, *Escherichia coli*, *Leuconostoc*, *Enterococcus* and *Lactobacillus*. Bacteriophage plaque sizes of 0.4-0.5 cm were demonstrated on the medium using a lactococcal bacteriophage.

INTRODUCTION

Species of the *Lactococcus, Pediococcus,* and *Leuconostoc* genera are important in the manufacture of fermented food products, including cheeses, buttermilk, yogurt, pickles and sausage (20, 22, 24). They are used for lactic acid production, pH lowering, the production of aroma compounds such as diacetyl, ripening of cheese and the production of substances which inhibit undesirable organisms (1, 4, 5, 6, 7, 10, 12). These genera are nutritionally fastidious and require a well-buffered medium for good growth. The dairy mesophilic lactic acid bacteria (LAB) are recognized as *Lactococcus lactis* subsp. *lactis* (LL), *L. lactis* subsp. *cremoris* (LC) and *L. lactis* subsp. *lactis* biovar. *diacetylactis* (LD) (16, 17). Several agar media are being used to growth these organisms (8, 19, 21, 22, 23). However, a selective medium for their isolation has not been documented. The objective of our research was to evaluate the efficiency of a new medium, Alsan, to recover *Lactococcus* spp. and to differentiate between those that utilize citrate and those negative for this trait.

MATERIALS AND METHODS

Cultures and activation:

Twenty-two strains from the *Lactococcus* genus were used (Table 5.1). Each culture was activated in M17 broth (Baltimore Biological Laboratory, Baltimore, MD) (BBL) at 30°C for 18-24 hr. Between transfers, strains were maintained in M17 agar keeps at 2-5°C.

Experimental medium and quantitative analysis:

The newly developed medium (Alsan) contained the following ingredients per liter of distilled water: Phenylethanol agar (Difco), 35.5 g; glycine anhydride (Sigma), 10g; lithium chloride (Sigma), 0.5g; sodium acetate anhydrous, 5g; potassium phosphate monobasic (J. T. Baker), 2 g; ammonium citrate dibasic (J. T. Baker), 2 g; arginine monohydrochloride (NB Corporation), 5g; bromocresol purple (Sigma), 0.02g; and α-lactose (Sigma), 5g. After autoclaving at 121°C for 15 min and tempering to 45°C in a water bath, 10 mL of 10% potassium ferricyanide solution and 10 mL of ferric citrate (FC)-sodium citrate (SC) solution (1g of FC + 1g of SC dissolved in 40 mL of distilled water) were added. The solutions were steamed for 30 min before being added to the tempered agar in order to detect citrate utilization (8). Also, 5 mL of filter sterilized solution containing 10 mg trimethoprim (Sigma) was added to each L of medium. The final pH of the medium as required for citrate permease induction (9, 13).was 5.9 ± 0.1. The other medium used for comparison was M17 (BBL) (21), a widely used medium for the cultivation of mesophilic LAB and their phages. Each active bacterium was diluted in 0.1% sterile peptone water to give about 10^4 to 10^5 colony forming units (CFU) per mL before surface plating onto media using the Spiral Plater System (Model D; Spiral System). Inoculated plates were incubated at 30°C for 48 h in a Gas-Pak (BBL) anaerobic System. Colonies were counted using the designed counting grid and at least three replicates, each in duplicate, were counted on each sample. Statistical analysis was conducted using the analysis of variance, WormStat™ program from Small Business Computers, Amherst, NH.

Mixed culture study:

Combinations of strains of LL, LD, and LC were mixed in sterile peptone water to give 10^4 to 10^5 CFU/mL before plating (Spiral System) onto M17 agar and Alsan. Plates were incubated at 30°C for 48 hr in a Gas-Pak and then examined for numbers and colony appearance.

Screening bacteria for growth on Alsan:

Several organisms (Table 5.2) were surface plated onto Alsan (Spiral Plater) after being activated in BHI broth or MRS broth and diluted in peptone water. Plates were incubated at 30°C or 37°C for 48 hr in a Gas-Pak except plates of *Pseudomonas aeruginosa* which were incubated aerobically. Control media for this phase of the study were M17 agar and Tryptic Soy Agar (TSA).

RESULTS AND DISCUSSION

Medium productivity and differential ability:

Colonies of LL appeared white or yellow against a yellow background though strains LM2306 and NCD0497 further metabolized arginine with resulting higher pH (Violet medium) due to ammonia production. On the other hand, LD grew as blue-green or blue colonies due to citrate metabolism and formation of ferric ferrocyanide (Prussion blue) (Figure 5.1). Color of the medium was either yellow or violet or a combination. Both LL and LD grew in the medium without addition of lactose, producing a strong violet color due to arginine metabolism and ammonia liberation. Comparison between the CFU/mL recovered of LL and LD on M17 and Alsan is shown in Table 5.1. Statistical analysis revealed no significant difference ($P>0.05$) between the two media. However, Alsan did not support the growth of LC strains (Table 5.1). An agar medium has been developed to differentiate LL and LC based on arginine metabolism (23). Also, a differential broth was formulated by Reddy et al., (13) to separate LL, LC and LD strains on the basis of citrate utilization and CO_2 production. Our further investigations revealed that LC205 and KH strains were inhibited by lithium chloride present in the Alsan medium. This is an important finding, though studies of the sensitivity of dairy starters to other inhibitors and antibiotics have been reported (11, 14). With one exception, all LL strains grew well on Alsan; strain C_2, however, was inhibited on the new medium (Table 5.1). Noteworthy in this regard was that Ribosomal RNA (rRNA) from strain C_2 also hybridized with 16S rRNA probes specific for LC (15). This indicated that LL C_2 was more closely related to LC than LL. All strains of LD grew as light blue or dark blue colonies due to citrate metabolism variants of LD. A medium previously described by Kemper and McKay (8) allowed differentiation between citrate positive and negative variants of LD. However, bacteria of other genera, such as *Leuconostoc* and *Lactobacillus*, are known for their utilization of citrate and production of diacetyl (2, 3, 8, 22). Citrate assimilation by such organisms can result in a false positive

identification as LD on KM agar. Other media have been developed for LAB (19, 22), but none were found selective for the mesophilic group. Furthermore, it was found that Alsan medium without bromocresol purple, ferricyanide, FC and SC supported bacteriophage plaque sizes of 0.4 - 0.5 cm when LL ATCC 7962 phage was tested. Other media were found to result in smaller plaque sizes (21).

Mixed cultures:

After incubation, colonies from mixed cultures of LL, LD, and LC developing on Alsan were of two types. The first was blue-green or blue in color indicating identity as LD. Yellow or white colonies were LL. In comparison with M17 plates, less dense growth was found on Alsan as a result of the inhibition of LC strains (data not shown), and the count of M17 minus the Alsan count provided an estimation of the LC count. Consequently, both LL and LD would be selectively enumerated in the medium when present along with LC in a mixed or multiple strain starter or product derived therefrom. This may decrease cost and expedite the time required to isolate pure cultures from strain mixtures. Furthermore, the absence of growth of LC on Alsan emphasizes additional differences that exist between group N lactococci to those known already (13, 19, 21, 22).

Selection effectiveness of Alsan:

Results indicated that Alsan completely inhibited *Leuconostoc*, *Streptococcus thermophilus* and *Lactobacilli* (Table 5.2). Such LAB may be present in cheeses and fermented milk along with LL, LC and LD. Consequently, Alsan can selectively allow growth of LL and LD to obtain pure cultures. In contrast, M17 which is not selective, allows the growth of all organisms tested. While *Escherichia coli*, *Pseudomonas aeruginosa*, and *Listeria monocytogenes* were completely inhibited by Alsan, *Enterococcus faecalis* and *Staphylococcus aureus* grew poorly and produced only pinpoint colonies which were hard to count. Interestingly, *Salmonella typhimurium* grew on the medium though Alsan contained

phenylethanol which is inhibitory to gram negative bacteria. The newly developed medium will be helpful in monitoring the growth of group N lactococci, especially when co-cultured with certain food spoilage and pathogenic organisms for evaluating their potency to inhibit such undesirable microorganisms. In this regard, it is well known that the inhibition of harmful organisms is dependent on the number of certain LAB present, as reported by Champagne et al., (4) Schaack and Marth (18) and Elliker et al., (7).

Table 5.1: Recovery of *Lactococcus* spp. on Alsan selective agar as compared to M17 agar[a]

Organism	Strain	Percent Recovery (%)
Lactococcus lactis	ATCC 7962	99
	LM 2306	101
	C_2	0
	F_2D_2	100
	NCDO 497	96
	ATCC 11454	100
	ATCC 11955	96
Lactococcus diacetylactis	18-16	99
	26-2	99
	DRC 1	98
	222	101
Lactococcus cremoris	P_2	0
	BK 5	0
	107/6	0
	205	0
	KH	0
	163	0
	196	0
	189	0
	819	0
	203	0
	224	0

[a]Count in M17 agar was considered 100%

Table 5.2: Selectivity of Alsan agar as indicated by the presence or basence of growth of selected bacteria after incubation at 30°C or 37°C for 48 hr.

Organism[a]	Growth on Alsan[b]
Streptococcus thermophilus CR5[b]	-
Leuconostoc cremoris J	-
Lactobacillus bulgaricus Y[b]	-
Lactobacillus isolate[b]	-
Pseudomonas aeruginosa ATCC 419	-
Escherichi coli V517	-
Listeria monocytogenes Scott A	-
Enterococcus faecalis CG 110	±[c]
Staphylococcus aureus	±
Salmonella typhimurium[b]	+

[a]Each organism had about 4->6 CFU (log_{10}) per mL when surface plated (Spiral plater) onto M17 or Tryptic Soy Agar.
[b]Incubation at 37°C for 48 hr.
[c]Only pinpoint colonies were found.
[d]+ = present, - = absent.

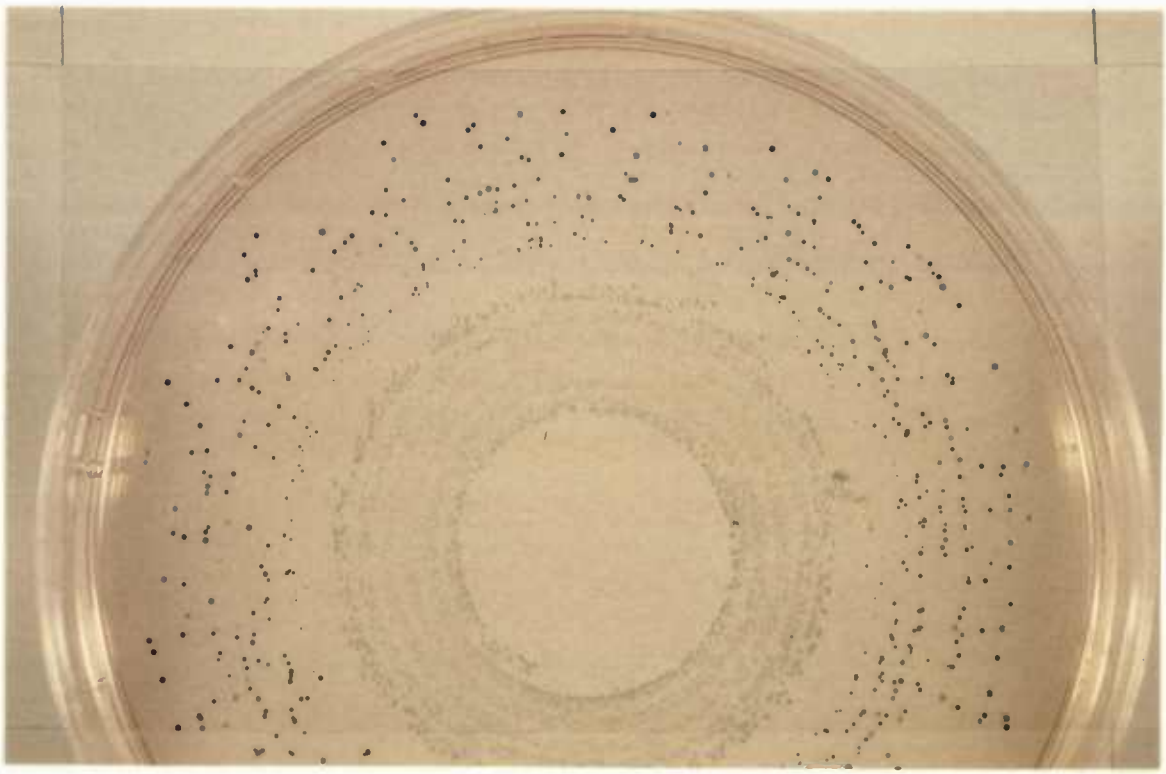
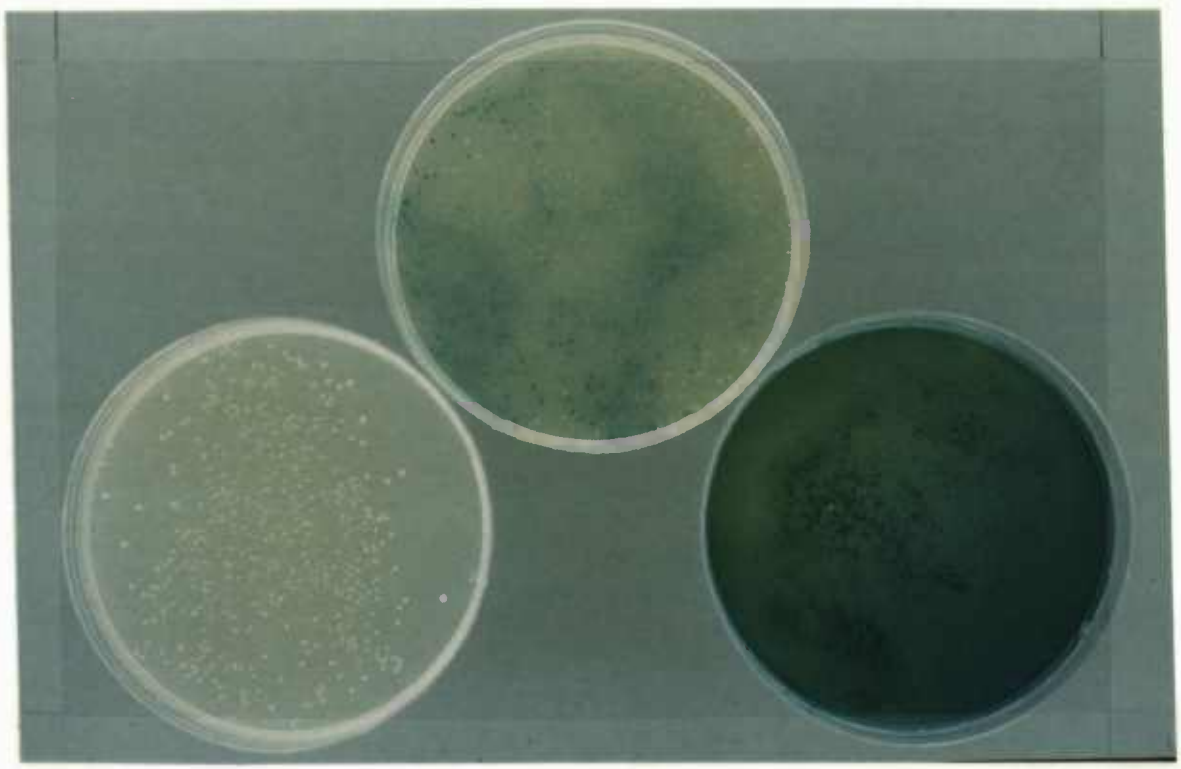


Figure 5.1: Appearance of colonies of *Lactococcus lactis* ATCC 7962 (white to grey in color) center area and *Lactococcus diacetylactis* 18-16 (light to dark blue in color) periphery on a spiral plate.

Addendum to Chapter 5

As added proof to the differential selectivity of Alsan medium, Figure 5.2 is presented with a photograph. This shows the appearance of *L. lactis* spp. *lactis* colonies (A), *L. lactis* spp. *lactis* biovar. *diacetylactis* colonies (B) and colonies developing from a mixture of these two bacteria (C). It may be seen that the two colony types are readily distinguishable, confirming the usefulness of this medium in recognizing these organisms when samples of various dairy products are plated thereon.

C



A B

Figure 5.2: Appearance of colonies of *Lactococcus lactis* spp. *lactis* (A), *Lactococcus lactis* spp.*lactis* biovar. *diacetylactis* (B) and a mixture of the two (C) growing on spread plates of Alsan medium incubated at 30°C for 48 hours.

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CHAPTER 6

Characterization of a *Lactobacillus* Isolate Inhibitory to Gram-Negative Bacteria

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ABSTRACT

Lactobacillus AS-1 is a gram-positive cocco-bacillary, catalase negative bacterium which does not produce hydrogen peroxide. The organism produces DL-lactic acid, deaminates arginine and grows over a wide range of temperatures (5°C - 45°C). It utilizes glucose, galactose, lactose and sucrose in addition to 17 other carbohydrates. Ethanol, CO_2, and acetic acid are produced indicating that the organism is heterofermentative. *Aeromonas hydrophila* and *Pseudomonas putida,* but not gram-positive bacteria, were sensitive to metabolites produced by AS-1 at pH 5 or below. The inhibitory substance(s) was heat stable, protease resistant and sensitive to pH values below 2. Three plasmids were detected in *Lactobacillus* AS-1 using a procedure recently developed in our laboratory. However, these plasmids were lost upon incubation at elevated temperatures(38°C-45°C) and this treatment yielded cured cells which were unable to ferment lactose. Cultures of the cured strain also were impaired in inhibitory activity against gram negative bacteria.

INTRODUCTION

The genus *Lactobacillus* has been divided into 3 subgenra, *Betabacterium, Streptobacterium* and *Thermobacterium* based on optimum temperature for growth and fermentation end products. Several approaches have been made to classify this heterogenous group (7, 16, 21, 26, 27, 28, 30). The genus *Lactobacillus* consists of gram positive rods and cocco-bacillary forms (7, 14, 16, 28, 30), which are catalase negative, non-spore forming and which possess homo or heterofermentative metabolic pathways. This genus was recently separated from similar gram positive rod shaped lactic acid bacteria now known as the *Carnobacterium* genus.Carnobacteria have meso-diaminopimelic cell wall peptidoglycan, produces only L-lactate and do not grow on acetate agar (21). Being lactic acid bacteria, lactobacilli are inhibitory to undesirable organisms by several mechanisms. These include nutrient competition, acid production, hydrogen peroxide accumulation, reduction of oxygen tension and production of specific inhibitory substances. Different bacteriocins and non-protein inhibitors produced by lactobacilli have been characterized in the literature (1, 8, 24, 25, 29).

The present study was designed to characterize a *Lactobacillus* isolate which was found antagonistic to gram negative bacteria in raw milk and milk products at refrigeration temperatures.

MATERIALS AND METHODS

Bacterial Isolate:

The *Lactobacillus* isolate was given the designation AS-1. The organism originally was misclassified as *Leuconostoc cremoris* and was in the Department of Microbiology culture collection without a particular strain designation. It was found by Al-Zoreky and Sandine to be inhibitory to some food spoilage and pathogenic bacteria in foods (5). The organism was kept frozen in cryoprotected nonfat dry milk (NDM) (2 ml of 11% NDM + 0.5 ml of 60% glycerol) at -20°C. Activation was carried out in MRS broth (Difco) at 30°C.by transferring the thawed milk culture to MRS broth with a minimum of transfers .

Morphological, Biochemical and Physiological Examinations:

Lactobacillus AS-1 was tested for morphology using gram stain and a wet mount technique. Catalase was tested by adding 3% aqueous H_2O_2 to colonies grown in MRS agar at 30°C for 30 hours, aerobically. Hydrogen peroxide assay was conducted in a diagnostic medium developed by Fontaine and Taylor-Robinson (13) and in milk by using the Perid test strips of Boehringer-Mannheim, Germany. Detection of cytochrome (Benzidine test) was by the procedure of Deibel and Evans (9.) Carbon dioxide production from glucose was monitored in an inverted Durham tube in MRS broth after incubation at 30°C for 48 hours. The API 50 CH system (API system, S. A. France) was used to assess carbohydrate metabolism. Pyrrolidonylarylamidase (PRYase) activity and deamination of arginine were determined in the API 20 Strep System. Gas chromatography (GC) analysis was used after growing the culture in MRS broth or 11% NDM with incubation at 30°C for 24 hours. Milk culture was acidified to pH 4.5 with hydrochloric acid to precipitate casein and the supernatant was filtered through Whatman paper and then a 0.45 µm filter.10 µl sample was injected into a Hewlett-Packard 5710A gas chromotograph. The GC column (Supelco, Inc.,

Bellefonte, PA) used consisted of 80/120 carbopack BAW 6.6% carbowax 20 M. The glass column had an O.D. of 1/4 inch and was 6 feet x 2mm I. D. Temperature of the oven was raised from 90°C to 200°C at 4°C/min. Nitrogen gas was the mobile phase at a flow rate of 20 ml/min. Temperatures of the FID and the injector were maintained at 250°C. Concentrations of volatile substances were quantitated using a Hewlett-Packard 3390A integrator calibrated with known concentrations of standard solutions of acetaldyhyde, ethanol, diacetyl, acetic acid, propionic acid and butyric acid. The organism was tested for growth at 5°C, 10°C, 25°C, 30°C, 37°C, 45°C, and 50°C by inoculating MRS broth (0.05% inoculum) from active culture followed by incubation at the assigned temperature for 48 hours, except at 10°C and below.when the incubation time was 7 days. Growth on Rogosa SL agar (Difco) at pH 5.5 and on Simmons citrate agar (Difco) also was assessed. Type of lactic acid produced from glucose in MRS broth culture (1:10 dilution of broth culture) was tested using the L-lactic acid kit (Catalogue No. 139084) and D-lactic dehydrogenase (Catalogue No. 736-970) of Boehringer-Mannheim GMBH, Germany. *Leuconostoc dextranicum* ATCC 19255 and *Enterococcus faecalis* CG 110 were used as known controls producing D-lactic acid and L-lactic acid, respectively. Sucrose agar described by Garvie (14) was employed to detect polysaccharide (dextran) production. *Leuconostoc dextranicum* was the positive control. Sensitivity to 30 µg vancomycin disks (Difco) was done according to the procedure of Orberg and Sandine (23).The organism was also tested for coagulation of milk (11% NDM) after incubation at 30°C for 24 hours from a 1% inoculum.

Detection of Bacteriocin Activity

Production of inhibitory substances was examined after growing *Lactobacillus* AS-1 in MRS broth plus 2% yeast extract at 30°C for 24 hours. Cells were removed by centrifugation at 8,000 x g for 15 minutes followed by filter sterilization (0.45 µm filter) 3 times. Growth metabolites were adjusted to different pH values (2, 5, 5.3, 6.5 and 7) using concentrated hydrochloric acid, or 3N sodium hydroxide. Control MRS broth was adjusted accordingly. Several test

organisms were activated in BHI broth (Difco) at 30°C or 37°C for 18 hours. Nutrient or MRS agar (Difco) tempered to 45°C then was inoculated with (0.2 - 0.5%) active culture and then plates were poured and left to solidify before making wells of 0.4 cm diameter. Test organisms were: *Pseudomonas putida*, *Aeromonas hydrophila* ATCC 7965, *Salmonella enteritidis* ATCC 4931 as well as *Escherichia coli* V517 and ATCC 23716. Gram positive bacteria were *Listeria monocytogenes* (Jalisco and Scott A), *Staphylococcus aureus* ATCC 13565, *Bacillus subtilus* 168, *Streptococcus thermophilus* CR5 *and Lactobacillus bulgaricus* Y. Yeasts were represented by *Candida lambica*, *Rhodotorula minuta* and *Zygosaccharomyces rouseil* After making wells in solidified, inoculated agar, 80 ul of either control broth or metabolites of *Lactobacillus* AS-1 was added to wells (deferred test). Plates were incubated at 30°C for yeasts and *Pseudomonas spp*, whereas pathogens were incubated at 30°C or 37°C for 18 hours. Inhibition Zones were measured (mm). Also, *Lactobacillus* AS-1 was grown in M17 medium (Difco), APT (BBL), BHI (Difco) and YM (Difco) broths, all with 2% yeast extract and tested for inhibition. Growth metabolites of *Lactobacillus* AS-1 (in MRS broth) also were tested for inhibition after heating at 121°C for 30 minutes, treatment with chloroform (25) or with several enzymes (1% concentration). Enzymes used were pronase E, pepsin, catalase, α-amylase, rennet, trypsin and lipase. All were Sigma products except lipase which was from Boehnringer-Mannheim, Germany.

Plasmid Profile:

Lactobacillus AS-1 was grown in MRS broth at 30°C for 18 hours and then transferred to 10 ml of lysis broth (3% inoculum) as described by Klaenhammer et al. (17). Growth was at 32°C for 2 hours (O.D. = 0.20 at 600 nm). Cells were harvested by centrifugation and plasmid DNA was extracted using the small-scale procedure of Anderson and McKay (6) with some modifications. Treatment with lysozyme (Sigma) was for 30 minutes at 37°C before lysis with SDS. Precipitation of DNA with isopropanol was at -20°C for at least 2 hours and electrophoresis (horizontal gel 15 cm x 10 cm) was in 0.8% agarose at 60 V for 2 hours. AS-1 was grown in MRS broth at 38°C,

40°C, 42°C, and 45°C for 24 hours before inoculating into lysis broth in advance of performing plasmid profile analyses using the procedure described above. Another plasmid profile procedure developed in our laboratory also was used. Harvested cells from lysis broth were treated with 100 µl chloroform and kept on ice for 10 minutes , after which cells were centrifuged for 10 minutes followed by removing chloroform by aspiration with the aid of a Pasteur pipette attached to a vacuum pump. The procedure of Anderson and McKay(6) was followed using mutanolysin (Sigma, M-9901) (50 units) at 37°C for 30 minutes instead of lysozyme. Sodium hydroxide (3.0 N) was added to the preparation with invertion the tubes 3 times to achieve thorough mixing. During all the procedures except lysis with mutanolysin, plasmid preparations were kept on ice. The rest of Anderson and McKay's procedure was followed as puplished. Both parental and the cured strains (at 45°C) were tested for inhibition against gram-negative bacteria of raw milk during cold storage.

RESULTS AND DISCUSSION

Biochemical and Physiological Characteristics:

Lactobacillus AS-1 was a gram positive cocco-bacillus, and negative for catalase, benzidine test, hydrogen peroxide production and blood hemolysis. In the literature, the cocco-bacillary *Lactobacillus* spp. were at one time misclassified as *Leuconostoc* due to their similar morphology and heterofermentative nature (16, 28, 30). The bacterium produced CO_2 from glucose (MRS broth) and utilized 21 of the 49 carbohydrates included in the API 50 CH system (Table 6.1). In comparison with *Lactobacillus confusus* ATCC 10881, a morphologically similar bacterium, *Lactobacillus* AS-1 metabolized 21 sugars whereas the other bacterium assimilated 16 sugars (Table6.1). Strain AS-1 deaminated arginine and was positive for PRYase in the API 20 Strep system. The previous two tests were used to differentiate between *Leuconostoc* and heterofermentative cocco-bacilli of the genus *Lactobacillus* (10). GC analysis (Fig6.1) revealed the production of ethanol and acetic acid by *Lactobacillus* AS-1, as well as the positive control *Leuconostoc dextranicum* ATCC 19255, confirming the heterofermentative nature of AS-1. *Enterococcus faecalis* did not produce ethanol since it is a homofermentative bacterium. Growth of the AS-1 isolate took place under all temperatures used except 50°C. It grew slowly at 5°C where visible turbidity was seen but only after 5 days. According to Ahn and Stiles (2) ,lactobacilli isolated from vacuum-packaged meat were able to grow at 5°C as well. The growth of the As-1 strain at lower temperatures therefore is not unusual and would be of advantage in restraining the proliferation of harmful organisms in refrigerated foods. Rogosa SL agar, (pH 5.5), a selective medium for lactobacilli, allowed growth of the test bacterium whereas no growth occured when citrate (Simmon's citrate slant) was the sole source of carbohydrate. Lactic acid, both L- and D-isomers, were produced by *Lactobacillus* AS-1. Production of DL-lactic acid, deamination of arginine and growth above 39°C separate lactobacilli from *Leuconostoc* (10,20,30). The fatty acid profile of the cell wall of

Lactobacillus AS-1 (performed by Accua lab., Inc., Newark, Delaware) did not match with that of *Leuconostoc* spp. (data not shown). Furthermore, polysaccharide (slime) was not produced by *Lactobacillus* AS-1 and the organism was sensitive to vancomycin. Vancomycin resistance in *Leuconostoc* spp. has been reported to be linked to plasmid DNA (10, 11, 23). The pH of 11% NDM inoculated with AS-1 after 18 hours of incubation at 30°C was 5. Thus it is evident that this bacterium is not a vigrous acid producer.

Screening *Lactobacillus* AS-1 For Inhibitor Production:

Table 6.2 shows typical inhibition zones against some gram negative bacteria. Gram positive bacteria were not inhibited (no inhibition zones), even after incubation for more than 10 hours. *Aeromonas hydrophila* and *Pseudomonas putida* were more sensitive to AS-1 metabolites than other gram negative bacteria tested. However, yeasts were not inhibited. Furthermore, no inhibition zones were detected against *Pesudomonas* spp. or *Aeromonas hydrophila* when the pH of AS-1 metabolites were adjusted to pH 5.1 or above (Table6.3). A similar pattern of inhibition was reported by Silva et al., (31), for a *Lactobacillus* GG and for *Propionibacterium shermanii* (3, 4). Furthermore, it was reported that *Lactobacillus bulgaricus* produced inhibitory substances against *Pseudomonas fragi* when the pH of fermented milk was 4 (1). In the present study. all gram positive bacteria tested, including the producing isolate (AS-1), were insensitive to AS-1 metabolites even at pH values below 5. An interesting finding was that *Listeria monocytogenes* growing at a distance of 10 mm around the wells of the filterate of *Lactobacillus* AS-1 tested catalase negative, in contrast to the growth on plates of *Staphylococcus aureus* or *Bacillus subtilis* Inhibition zones against *Pseudomonas* spp were only seen when AS-1 was grown in MRS or APT broth while AS-1 grown in BHI, M17 or YM broth (at pH 4.5) did not cause zones of inhibition (Table 6.4). Also, control MRS broth did not cause inhibition zones. Filtrate inhibitor(s) of *Lactobacillus* AS-1 from MRS broth was heat stable (121°C for 30 minutes) and not antagonized by proteases, chloroform or other enzymes tested (Table6. 3). Similarly, antimicrobial materials produced by

Lactobacillus GG was reported by Silva et al. (31) to withstand both heating and treatment with several enzymes. Interestingly, filtrates of *Lactobacillus* AS-1 loward to pH 2 and left for 24 hours at 5°C or heated at 121°C for 30 minutes before being raised to pH 4.5 (with 3N NaOH) and assayed against *Pseudomonas putida* gave smaller inhibition zones (>50% reduction in diameter) in comparison with filterates kept at pH 4.5 or 7. However, filtrates heated at 121°C for 30 minutes at pH 12, cooled and adjusted to pH 4.5 gave larger inhibition zones. The increase in zone diameters may be the result of Maillard reaction products scince it has been reported these products are inhibitory to selected bacteria (32).

Plasmid Detection and Curing:

Plasmid DNA profiles showed that *Lactobacillus* AS-1 contains 3 plasmids (4, 10, and 23 Kb) when analyzed using the chloroform-mutanolysin procedure. However, the Anderson and McKay procedure (Fig.6.2) failed to detect these plasmids. Other researchers have reported the ineffectiveness of lysozome in digesting the cell wall of some gram positive bacteria (12, 18). The combination of chloroform and mutanolysin facilitated the lysis process and revealed the plasmids not detected by the other procedure. Our procedure was also successful in identifying plasmid DNA of members of the *Lactococcus* and *Leuconostoc* genera (data not shown). When *Lactobacillus* AS-1 was grown at temperatures at or above 38°C for 24 hours, all three plasmids were lost (Fig.6.2). In addition, carbohydrate metabolism profiles (API 50 CH system and API 20 Strep system) showed that the parental strain and the cured one (45°C) were identical with the exception of lactose which was negative in the cured isolates. Loss of the lactose plasmid using elevated temperatures in lactic acid bacteria has also been reported in the literature (15, 17). Loss of lactose metabolism renders the plasmid-free mutant ineffective in inhibiting gram negative bacteria in refrigerated milk, since lactose is the only carbon source available under these conditions. Since CsCl plus ethidium bromide centrifugation step was not carried out, the three bands on the gel may be different forms of the same plasmid which encoded for

lactose utilization and this plasmid may have been lost by elevated temperature (38-45°C). As a result, mutants were unable to grow in raw milk and gave the same inhibition as was the case with the parental strain.

Table 6.1: Carbohydrate assimilation patterns by *Lactobacillus* AS-1 and *Lactobacillus confusus* ATCC 10881 using the API 50 CH system after incubation at 30°C for 48 hours.

Acid production From	Strain	
	AS-1	ATCC 10881
L. arabinose	++	-
Ribose	++	++
D-xylose	-	++
Galactose	++	++
Glucose	++	++
Fructose	++	++
Mannose	++	++
Mannitol	+	-
N-acetylglucoseamine	++	++
Amygdalin	+	++
Arbutin	+	++
Esculin (hydrolysis)	++	++
Salicin	+	+
Cellibiose	++	++
Maltose	++	++
Lactose	++	-
Mellibiose	+	-
Sucrose	++	-
Trehalose	++	-
Starch	++	-
Glycogen	++	-
Gentibiose	+	+
Gluconate	-	+
2-Ketogluconate	-	+

++ Strongly positive
+ Moderately positive
- Negative

Also, both strains were negative for the following carbohydrates: Glycreol, Erythritol, D-Arabinose, L-xylose, Adonitol, b-methylxyloside, Sorbose, Rhamnose, Dulcitol, Inositol, Sorbitol, α-methylmannoside, α-methylglucoside, Inulin, Melezitose, Raffinose, Xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol and 5-ketogluconate

Table 6.2: Deferred antagonistic activity of *Lactobacillus* AS-1 (measured by diameter of inhibition zones) against several microorganisms in nutrient agar or MRS agar.

Test Organism	Inhibition Zone (mm)
Gram Negative Bacteria:	
Aeromonas hydrophila	6
Pseudomonas putida	6.5
Escherichia coli	1
Salmonella enteritidis	2
Gram Positive Bacteria:	
Bacillus subtilus	0.0
Listeria monocytogenes	0.0
Staphylococcus aureus	0.0
Streptococcus thermophilus	0.0
Lactobacillus bulgaricus	0.0
Lactobacillus AS-1 (producer)	0.0
Yeast (3 genera)	0.0

Table 6.3: Effects of enzymes, pH, heating and chloroform treatments on the inhibitory substance(s) produced by *Lactobacillus* AS-1 against *Aeromonas hydrophila* ATCC 7965 or *Pseudomonas putida* W.

Treatment	Presence (+) or Absence (-) of Inhibition Zones Against the Test Bacteria
pH 4.5 with heating at 121°C for 30 minutes	+
pH 2 at 5°C for 24 hours or at 121°C for 30 minutes and the adjusting to pH 4.5	-
pH adjustments >5.1	-
Proteinases, Amylase, Lipase and Catalase	+
Treatment with chloroform	+

Table 6.4: Production of Inhibitiory Substance(s) by *Lactobacillus* AS-1 in Several Growth Media

Growth Medium	Inhibition Results*
MRS broth (Difco)	+
APT broth (BBL)	+
M-17 (BBL) and Glucose	-
BHI broth (Difco)	-
YM broth (Difco)	-

*Tested against *Aeromonas* or *Pseudomonas* in nutrient agar.

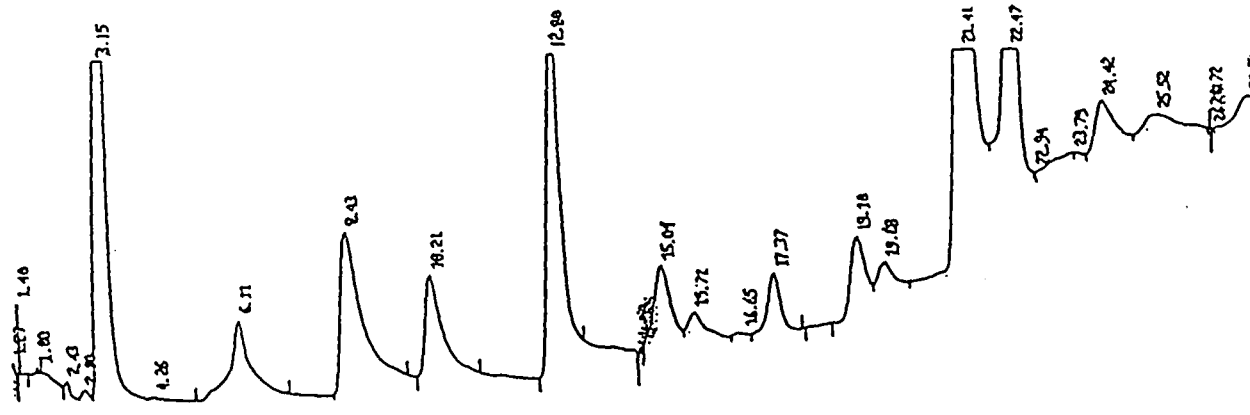

Figure 6.1: GC analysis of fermentative products of *Lactobacillus* AS-1 in 11% non-fat dry milk after incubation at 30°C for 24 hours. Peaks at retention times 3.15 minutes and 12.80 minutes indicated the identity of ethanol (214 ppm) and acetic acid (331 ppm), respectively.

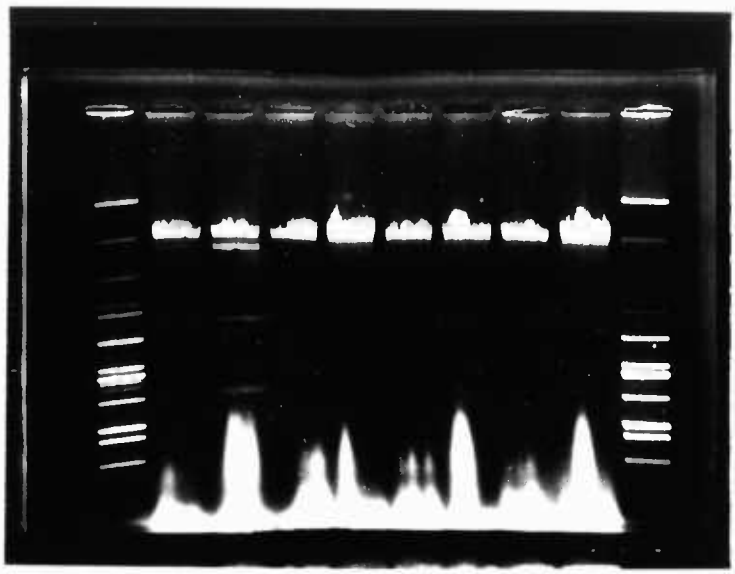

Figure 6.2: Plasmid DNA profile of *Lactobacillus* AS-1 and its mutants in 0.8% Agarose gel electrophoresis at 60 volts.

Lane 1 and 10, *E. coli* V517 mobilitystandard Lane 2, AS-1 (parental) using Anderson- McKay procedure
Lane 3, AS-1 (Parental) this study procedure
Lane 4-9, AS-1 (Mutants) cured at 38°C (lane 4-5), 40°C (lane 6-7), and 42°C (lane 8-9) for 24 hours using either procedure

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SUMMARY

Raw milk was inoculated with 10^2 CFU/ml of *L. monocytogenes*, *Staphylococcus aureus*, *Salmonella* spp. or *E. coli* and 10^7-10^8 CFU/ml of different lactic acid bacteria (LAB). *Lactococcus lactis* subsp. *lactis* ATCC 7962 or *Pediococcus pentosaceus* A eliminated *L. monocytogenes* from raw milk held at 5°C for less than a week due to production of bacteriocins. Incubating raw milk at 5°C caused elimination of *Staphylococcus aureus* when co-cultured with *Lactobacillus bulgaricus*. Gram-negative bacteria *Salmonella*, *E. coli* and *Pseudomonas* spp. were inhibited in refrigerated raw milk containing *lactobacillus* AS-1. This strain produced inhibitory substance(s) which were heat stable (121°C for 30 min.), pH sensitive, resistance to cleavage by proteases, and only produced in some growth media.

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