Medical Journal of the
Islamic Republic of Iran

Volume 10
Number 2
Summer 1375
August 1996

GROWTH INHIBITION OF PATHOGENS BY LACTIC ACID BACTERIA PRODUCING INHIBITORY SUBSTANCES

FARROKH A. NAKHDJAVANI, NASRIN MOAZAMI, Ph.D.,* AND HASSAN LAMEA, Ph.D.**

*From the Department of Microbiology, Medical Science Faculty, Tarbiat Modarres University, Tehran, the *Department of Biotechnology, Iranian Research Organization for Science and Technology, P.O. Box 15815-3338, Tehran, and the **Food Science and Technology Center, Postgraduate and Advanced Research Branch, Islamic Azad University, P.O. Box 19395-1775, Tehran, Islamic Republic of Iran.*

ABSTRACT

71 samples of milk and dairy products were collected from 18 cities throughout Iran. 93 strains of lactic acid bacteria (LAB) were isolated and assayed against four opportunistic pathogens including *E. coli*, proteus and klebsiella as gram-negative and *S. aureus* as a gram-positive organism. All indicators (organisms) were hospital strains. Among isolated bacteria, lactobacilli (37 isolates) had stronger activity against gram-negative pathogens. 37 isolates were lactococci out of which 9 strains were leuconostocs and seven strains were *S. faecalis*. *S. aureus* was more sensitive to lactococci than lactobacilli. Only one isolate of leuconostoc inhibited the growth of gram-negative indicators, but five isolates inhibited *S. aureus*. The use of bacteriocin-producing lactic acid bacteria in dairy products provides a natural mean of preservation.

Keywords: Lactic Acid Bacteria, Bacteriocin, Growth Inhibition, Antibiosis.

MJIRI, Vol. 10, No. 2, 159-163, 1996.

INTRODUCTION

The antimicrobial properties of lactic acid bacteria (LAB) are of special interest to researchers involved in developing strongly competitive starter-cultures for food fermentation. Antibiosis is an antagonistic phenomenon between micro-organisms to the detriment of one of them.[1] Metchnikoff in 1908 first suggested that ingestion of yoghurt was beneficial and could prolong life.[4] He postulated that intestinal bacteria produce toxic amines, and that the harmful effects of these putrefactive organisms could be minimized or prevented by establishing the proper lactobacillus flora in the gut. Lactose in yoghurt with live bacteria is better tolerated than lactose in milk, partly because of the activity of beta-galactosidase (beta-gal),[23] which digests lactose *in vivo*.

In the present investigation, the product potential for bacteriocin-like substances in Iranian starter-cultures was determined.

* Head of the Biotechnology Center, Iranian Research Organization for Science and Technology, P.O. Box 15815-3338, Tehran, I.R. Iran.

** Head of the Food Science and Technology Center, Postgraduate and Advanced Research Branch, Islamic Azad Univ., P.O. Box 19395-1775, Tehran, I.R. Iran.

MATERIALS AND METHODS

Sampling

71 samples of milk and dairy products were collected from 18 cities chosen from five states throughout Iran.

Media

The following media were used: M16 agar and broth[10] for

Table I. Identification of milk and dairy isolates.

Source and Comments							
No.	**Isolates**	**Genus and Isolates**	**Cheese**	**Yoghurt**	**Milk**	**Cream & Butter**	**Total**
1	L. C	*Lactobacillus casei*	10	1	1	6	18
2	L. CR	*Lactobacillus casei rhamnos*	1	1	--	----	2
3	L. L	*Lactobacillus lactis*	4	1	1	-----	6
4	L. B	*Lactobacillus bulgaricus*	1	3	--	-----	4
5	L. A	*Lactobacillus acidophilus*	4	1	--	-----	5
6	L. H	*Lactobacillus helveticus*	--	1	--	-----	1
7	L. Sp	*Lactobacillus sp.*	1	---	--	-----	1
8	S. L	*Streptococcus lactis*	12	3	8	1	24
9	S. D	*Streptococcus diacetylactis*	2	4	2	-----	8
10	S. T	*Streptococcus thermophilus*	4	2	--	-----	6
11	S. F	*Streptococcus fecalis*	5	1	1	-----	7
12	Leu. C	*Leuconostoc cremoris*	2	1	1	2	6
13	Leu. sp	*Leuconostoc sp.*	2	---	1	-----	3
Total			48	19	15	9	91

isolation of lactococci, MRS broth[11] for carbohydrate fermentation, Rogosa agar[11] for isolation of lactobacilli, blood agar and Muller-Hinton agar for detection of growth inhibition. A modified medium which distinguished between citrate-fermenting and noncitrate-fermenting species of lactococci,[13] SF medium for fecal streptococci,[14] skim milk for maintenance of the organisms as frozen stocks, and LB (lactose broth) for gas detection from carbohydrate fermentation were also used.

Indicator organisms

For strains of pathogenic bacteria isolated from different specimens such as wound, abscess, urine and feces were obtained from the Bacteriology Department of the Central Laboratory of Imam Khomeini hospital.

Screening for antagonistic activity

Overnight cultures of LAB were spotted onto agar plates. The plates were incubated for 18 h at 30°C (for lactococci and leuconostocs) and 37°C (for lactobacilli) to allow colonies to develop. Soft agar (3.5 ml; 0.7%), inoculated with 10 cells of the indicator organism in the late exponential phase of growth, was then poured onto the surface. The plates were checked for zones of inhibition surrounding the lactic acid bacteria colonies after incubation at 30 and 37°C for 6, 12 and 24 h. Inhibitory activity due to the action of hydrogen peroxide was excluded by the addition of catalase (5 mg/ml, Sigma; in 10 mM phosphate buffer, pH=7),[16] or

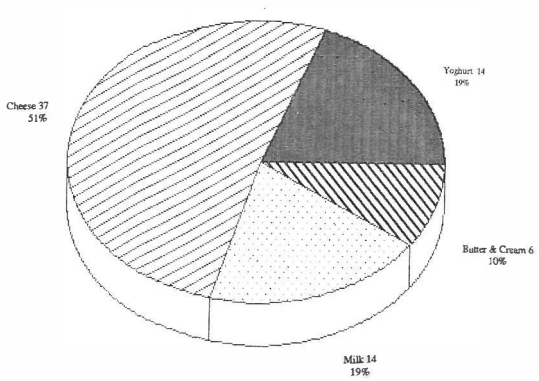

Fig. 1. Frequency of dairy samples.

by covering the LAB colonies with MRS agar medium. Cultures were neutralized by addition of 5 N NaOH to exclude the organic acids before assayed against indicators.[5]

RESULTS

The frequency (type) of dairy samples are shown in Fig. 1, and the frequency of LAB in Figs. 2,3. With the exception of miscellaneous bacteria such as seven strains of *S. faecalis* which were identified and separated via SF medium,[14] lactic strains (LAB) were identified as the genus of lactobacilli (many species such as: *Lb. delbrueckii*, subsp. *bulgaricus*, *Lb. delbrueckii*, subsp. *lactis*, *Lb. acidophilus*, *Lb. helveticus*,

Table II. Physiological characteristics of lactic acid bacteria.

Characteristics	Strain Designation									
	L. C	L. CR	L. L	L. B	L. A	L. H	S. L	S. D	S. T	Leu.C
Fermentation of:										
Esculin	w	+	-	-	+	-	+	+	-	-
Glucose	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	d
Lactose	+	+	+	+	+	+	+	+	+	d
Maltose	+, -	+, -	+	-	+	+	+	+	-, +	d
Mannitol	+	+	-	-	-	-	+	+	-	-
Mannose	+	+	+	-	+	w	+	+	+	-
Raffinose	-	-	-	-	+, -	-	-	-	-	-
Rhamnose	-	+	-	-	-	-	-	-	-	-
Sorbitol	+	+	-	-	-	-	-	-	-	-
Sucrose	+, -	+, -	+	-	+	-	+	+	+, -	+
Slime from sucrose	-	-	-	-	-	-	-	-	-	+
Gas from glucose	-	-	-	-	-	-	-	+, -	-	+
Growth at:										
15°C	+	+	-	-	-	-	+	+	-	+
40°C	+, -	+	+	+	+	+	+	+	+	-
45°C	-	+	+	+	+	+	-	-	+	-
50°C	-	-	+	+	+	+	-	-	+	-
Growth in:										
(Final % of NaCl)	>6.5	>6.5	>6.5	>6.5	>6.5	>6.5	4-6	4-6	>2	>3
Gram stain	+	+	+	+	+	+	+	+	+	+
Morphology	r	r	r	r	r	r	c	c	c	c
Catalase test	-	-	-	-	-	-	-	-	-	-
Final pH in skim milk	>4	>4	>4	>4	>4	>4	4.5	4.5	4.5	5

d: Delayed reaction; r: Rod; c: Cocci.[15, 27, 28]

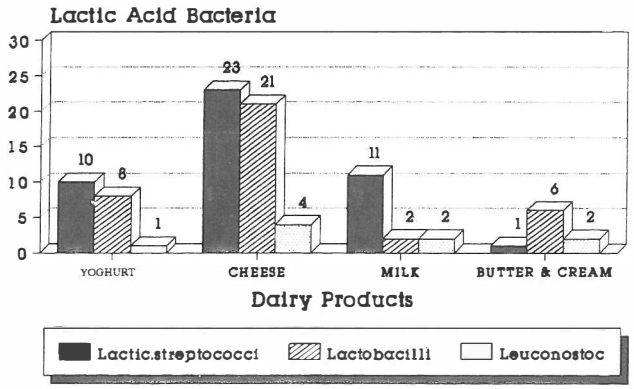

Fig. 2. Frequency of lactic acid bacteria in milk and dairy products.

Lb. casei, subsp. *casei*, *Lb. casei*, subsp. *casei rhamnosus*, ...) and the genus of lactococci (lactic streptococci) such as *Lac. lactis*, subsp. *lactis*, *Lac. lactis*, subsp. *diacetylactis* and species of *Leuconostoc mesenteroides*, subsp. *cremoris*. Leu. spp. are listed in Table II.

All lactic strains (Fig. 3) were assayed for bacteriocin-like antagonism against four kinds of indicator strains. The indicators were selected as the most resistant representative for usual antibiotics. Twenty strains of lactobacilli (22%) inhibited the growth of three kinds of gram-negative indicators (55 cases, # 60).

Concerning lactococci, approximately one-sixth of the strains (six strains = 7%) showed antagonism against gram-negative indicators. 27 of these lactic cultures (71%) inhibited the growth of *S. aureus*, whereas 17 strains of lactobacilli were active against *S. aureus* (46%). Gram-negative indicators were sensitive to one strain of leuconostocs and *S. aureus* was sensitive to four strains of these lactic cultures (Fig. 4).

DISCUSSION

The present study was undertaken to estimate the frequency of bacteriocin-like inhibitory substances by Iranian types of LAB, isolated from milk and other dairy products.

A majority (54%) of LAB demonstrated inhibitory activity against gram-positive indicators and 30% of isolated lactic cultures inhibited gram-negative indicators. These results are acceptable in comparison with bacteriocin production in different studies (DeKlerk, 1967: bacteriocinogeny in *Lb. fermenti* toward four kinds of lactobacilli was 15.5%.[20] Barefoot & Klaenhammer, 1983,

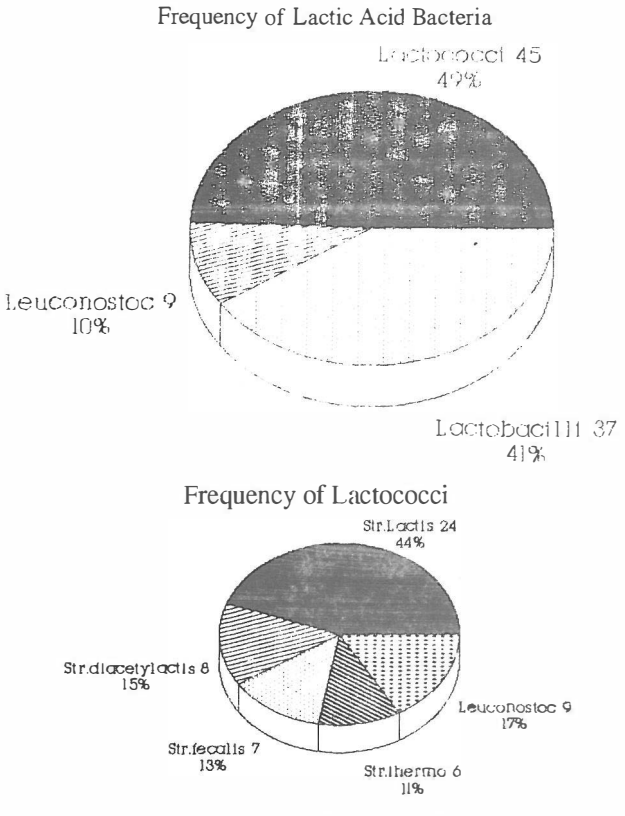

Fig. 3. Frequency of lactic acid bacteria.

demonstrated 63% inhibitory activity by *Lb. acidophilus* against four kinds of lactobacilli). Oxford, A.E.[1] extracted an inhibitory agent named diplococcin from two cultures of lactic streptococci (lactococci) that had variable effects on *S. aureus* and no action on *E. coli*, but our lactococci (*Lac. lactis, Lac. lactis* subsp. *diacetylactis*) had strong activity against *S. aureus* (70%) and moderately inhibited *E.coli* (45%). In contrast to previous reports,[4] this inhibition could not be attributed to the production of lactic acid or acidification because of the buffering of the medium. Covering the colonies with a drop of MRS agar immediately after anaerobic incubation avoided the production of H_2O_2.

Exposure of the LAB colonies with UV did not affect inhibition. These experimental precautions enabled us to attribute the inhibition to the production of bacteriocin-like substance(s).

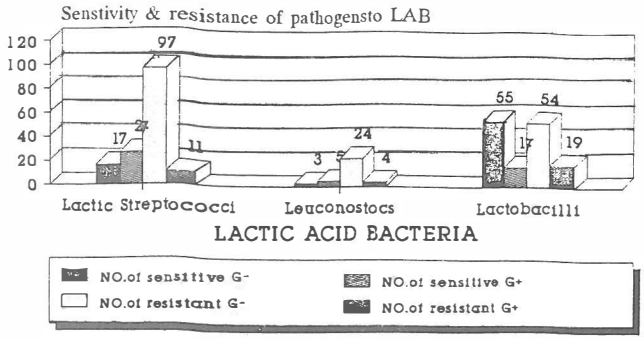

Fig. 4. Antibiosis–growth inhibition of pathogens by lactic acid bacteria.

Several studies have shown that lactobacilli constitute one of the dominant groups of intestinal and fecal organisms being at levels between 10 and 10.[26]

Gonzalez et al. (1990) have shown that lactobacilli are present in feces at levels of 10 and 10/gm when fermented milk is consumed.[19,27] The sensitivity of gram-positive and -negative indicators to our isolated lactic cultures in comparison to bacteriocin production in different studies,[4,12,16] indicates the uniqueness of the bacteriocin produced by these cultures in or on various substances such as meat.[12]

Many dairy products depend upon the microbiological activities of starter-cultures, particularly those cultures containing *Lactococcus lactis*, subsp. *diacetylactis*, lactobacilli and leuconostocs, to control the growth of spoilage microorganisms.[8,12,16,23,24] Most isolated strains of lactococci were *Lactococcus lactis.* The most part of *Lac. lactis*, subsp. *diacetylactis and Leuconostoc cremoris* was isolated from cream, butter and raw milk. Two French cream cheeses (Piccon, Novelle) were tested, but isolated lactic cultures failed to show any growth inhibition of pathogens. Our study revealed that the growth inhibition of gram-negative pathogens by lactobacilli was much stronger than lactococci; in contrast, growth inhibition of *S. aureus* by lactococci was better than lactobacilli. The nutritional and therapeutic aspects of LAB, particularly lactobacilli, for anticholesterolemic and anticarcinogenic effects[21] must be emphasized.

Diacetyl is a factor other than antibiotics or acids, produced by citrate-fermenting bacteria (*Leuconostoc cremoris* and *Lactococcus lactis*, subsp. *diacetylactis*) which contributes to the ability of these cultures to exert antagonistic action toward undesirable microorganisms. Diacetyl is inhibitory to both food-borne pathogens and spoilage microorganisms.[24,25] Of the inhibitory substances produced by lactococci, Nisin has received the greatest attention as a food additive to retard the growth of spoliage microorganisms, because of its heat resistance. Acidolin, Acidophilin and Lactocidin are produced from various strains of *Lb. acidophilus*.[19,20,22] Lactocidin is reportedly produced only on solid laboratory media and cannot be

isolated from liquid media in which the organisms have grown. Due to the nature of the isolation procedure, the bacteriocin-producing laboratory isolates grow well at refrigeration temperature, which could give them a competitive edge over spoilage organisms.[17,18] However, if milk and dairy products are contaminated with psychrotrophic food-borne pathogens, these products could pose a serious health threat. The use of bacteriocin-producing microorganisms may thus provide a natural mean of preservation. However, additional studies are needed to precisely characterize the inhibitory metabolites produced by lactic acid bacteria.

REFERENCES

1. Babel FJ: Antibiosis by lactic culture bacteria. Purdue Univ. West Lafayette47907 Journal Paper No. 6475. J Dairy Science 60 (5): 815-820, 1976.
2. Martini MC, Lerebours EC, Lin WJ, Harlander SK, Berrada NM, Antoine JM, Savaiano DA: Strains and species of lactic acid bacteria in fermented milks (yogurts): effect on *in vivo* lactose digestion. Am J Clin Nutr 54: 1041-6, 1991.
3. McGill K, et al: *In vivo* digestion of yogurt lactose by yogurt lactase. Nutrition Rev 42 (6): 216-17, 1984.
4. Barefoot SF, Klaenhammer TR: Detection and activity of Lacacin B, a bacteriocin produced by *Lactobacillus acidophilus*. Appl Environ Microbol 45 (6): 1808-15, 1983.
5. CarminatiD, GiraffaG, Bossi MG: Bacteriocin-like inhibitors of *Streptococcus lactis* against *Listeria monocytogenes*. J Food Protection 52 (9): 614-617, 1989.
6. Vaughan EE, Daly C, Fitzgerald GF: Identification and characterization of helveticin V-1829, a bacteriocin produced by *Lactobacillus helveticus* 1829. J Appl Bacteriol 73: 299-308, 1992.
7. Lindgren SE, Dobrogosz WJ: Antagonistic activities of lactic acid bacteria in food and feed fermentations. FEMS Microbiol Rev 87: 149-173, 1990.
8. Hastings JW, Stiles ME: Antibiosis of *Leuconostoc geldium* isolated from meat. J Appl Bacteriol 70: 127-134, 1991.
9. Hurst A: Nisin. Advances in Appl Microbiol 27: 85-123, 1981.
10. Elliker PR, Anderson AW, Hannesson G: An agar culture medium for lactic streptococci. J Dairy Sci 39: 1611-12, 1956.
11. DeMan JC, Rogosa M, Sharpe ME; A medium for the cultivation of lactobacilli. J Appl Bacteriol 23: 130-135, 1960.
12. Lewus CB, Kaiser A, Montville TJ: Inhibition of food-borne bacterial pathogens by bacteriocins from lactic acid bacteria isolated from meat. Appl Environ Microbiol 57 (6): 1683-8, 1991.
13. Kempler GM, McKay LL: Improved medium for detection of citrate-fermenting *Streptococcus lactis* subsp. *diacetylactis*. Appl Environ Microbiol 39 (4): 926-27, 1980.
14. Hanney CL, Norton IL: Enumeration, isolation and study of fecal streptococci from river water. Proceedings of the Society for Appl Bacteriol 1: 39, 1947.
15. Kandler O, Weiss N: Regular nonspore-forming rods (lactobacilli). In: Sneath PHA (ed), Bergey's Manual of Systematic Bacteriology. Baltimore: Williams and Wilkins, pp. 1708, 1985.
16. Geis A, Singh J, Teuber M: Potential of lactic streptococci to produce bacteriocin. Appl Environ Microbiolp: 205-211, Jan. 1983.
17. Sulzer G, Busse M: Growth inhibition of *Listeria* spp. on camembert cheese by bacteria producing inhibitory substances. International J Food Microbiol 14: 287-296, 1991.
18. Chateau N, Castellanos I, Deschamps A M : Distribution of pathogen inhibition in lactobacillus isolates of a commercial probiotic consortium. J Appl Bacteriol 74: 36-40, 1993.
19. Apella M C , Gonzalez S N, Maria E, Nador de Maci AS, Romeron N, Oliver G: *In vitro* studies on the inhibition of *Shigella sonnei* by *Lactobacillus casei* and *Lact. acidophilus*. J Appl Bacteriol 73: 480-483, 1992.
20. De Klerk HE: Properties of a *Lactobacillus fermenti* bacteriocin. J Gen Microbiol 48: 309-316, 1967.
21. Gilliland SE: Bacterial Starter Cultures for Foods. CRC Press, Inc., Boca Raton, Third Printing, 1988.
22. Schillinger U, Luckefk: Antibacterial activity of *Lactobacillus sake* isolated from meat. Appl Environ Microbiol 55: 1901-6, 1989.
23. Lawrence RC,Thomas TD, Lerzaghi BE: Reviews of the progress: cheese starters. J Dairy Res 43: 141-193, 1976.
24. Jay JM: Antimicrobial properties of diacetyl. Appl Environ Microbiol 44: 525, 1982.
25. Shahani KM, Ayebo AD: Role of dietary lactobacilli in gastrointestinal microecology. Am Clin Nutr 33: 2448-57, 1980.
26. Gonzalez S, Albarracin G, Locacio De Ruiz M, Male M, Apella MC, Pesce De Ruiz Holgado A, Oliver G: Prevention of infantile diarrhoea by fermented milk. Microbiologic-Aliments Nutr 8: 349-354, 1990.
27. Orvin Mundt J: Lactococci. In: Sneath PHA, (ed), Bergey's Manual of Systematic Bacteriology. Baltimore: Williams and Wilkins, pp. 1065, 1985.
28. Garvie E: Genus Leuconostoc. In: Sneath PHA, (ed), Bergey's Manual of Systematic Bacteriology. Baltimore: Williams and Wilkins, pp. 1071, 1985.