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Antimicrobial Activity and Characteristics of Bacteriocins Produced by Vaginal Lactobacilli

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Abstract: The aim of this study is to test the production of bacteriocin in vaginal Lactobacilli and determining the characterization and antibacterial activity of this bacteriocin. For this reason 100 *Lactobacillus* strains were isolated and identified from vaginal swab samples of 75 women who attended obstetric and gynecology clinics. It was determined that six of 100 *Lactobacillus* strains produced bacteriocin. The antibacterial activity of bacteriocins was examined and found to be effective on vaginal *L. gasseri, L. acidophilus, Gardnerella vaginalis* ATCC 14018 and *Pseudomonas aeroginosa* ATCC 10145. We also found that bacteriocins were sensitive to proteinase enzymes and alkalinity, but resistant to catalase. Two of them, TL059a and TL080, were resistant to chloroform and 1 h boiling. It was observed that adding 1% NaCl to medians increased bacteriocin production and it was also found that mitomycin C induced Rogosa SL media was more suitable than MRS ($Z = -2$, $p < 0.05$).

Key Words: *Lactobacillus*, Bacteriocin, Antibacterial activity

Introduction

Among lactic acid bacteria members, the lactobacilli are composed of a diverse group of homofermentative and heterofermentative species. The production of bacteriocins by lactic acid bacteria has been known for many years (1). According to the original definition, the term bacteriocin refers to proteins of the colicin type, characterized by lethal biosynthesis, intraspecific activity, and adsorption to specific receptors. Those produced by gram-positive bacteria fit closely to the classical colicin model (2). *Lactobacillus* bacteriocins are found within each of the four major classes of antimicrobial proteins produced by lactic acid bacteria. Class I (lantibiotics) was only recently discovered in the *Lactobacillaceae* by Mordvedt et al. (3). Lantibiotics are small membrane-active peptides (<5 kDa) containing the unusual amino acid lanthionine. Class II; small heat-stable, non-lanthionine containing membrane-active peptides (<10 kDa) characterized. The class III bacteriocins, which have to date only been found in *Lactobacillus*, include heat-labile proteins of large molecular mass. Class IV is a complex bacteriocin group. These proteins are associated with other lipid or carbohydrate moieties, which appear to be required for activity. The bacteriocin was relatively hydrophobic and heat stable (1).

Lactobacilli produce many different bacteriocins of similar activity, and are usually predominant species in the vaginas of healthy women. Lactobacilli play an important role in maintaining vaginal health. They produce lactic acid and H_2O_2, which can prevent the overgrowth of other microorganisms in the vagina including *E. coli* and *G. vaginalis* (4,5).

The purpose of this study was to investigate the antibacterial activity of *Lactobacillus* strains of vaginal origin. Furthermore, we sought to characterize bacteriocins for their structural properties and determine their antimicrobial activities against some common human pathogens including *Gardnerella vaginalis*, *Pseudomonas aeroginosa*, *Proteus vulgaris*, *Escherichia coli*, *Enterobacter cloacea*, *Streptococcus milleri*, *Staphylococcus aureus* and *Candida albicans*.

Materials and Methods

Bacterial strains, media and growth conditions: *Lactobacillus gasseri* ATCC 9857, *L. fermentum* ATCC 14931, *L. fermentum* ATCC 23271, *L. delbrueckii* subsp. *delbrueckii* ATCC 9649, *L. delbrueckii* subsp. *lactis* ATCC 4749, *L. jensenii* ATCC 25258, *L. casei* subsp. *casei* ATCC 27139, *L. vaginalis* ATCC 49540 and *L. acidophilus* ATCC 4356, *Streptococcus milleri* IS51 and *G. vaginalis* ATCC 14018 were provided by Tao at the University of Illinois in Chicago. *Lactobacillus* type strains were used for species identification as control strains. *Escherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 13315, *Pseudomonas aeruginosa* ATCC 10145, *Enterobacter cloacae* ATCC 13047, *Staphylococcus aureus* ATCC 25923, and *Candida albicans* ATCC 60193 were obtained from the Hifzisihha Institute of Refik Saydam (Ankara, Turkey).

The vaginal swab samples transported in Thioglycollate Broth (Difco, Detroit, MI) medium were inoculated onto Rogosa SL agar (Difco) and incubated at 37 ºC in a candle jar for 48 h. The lactobacilli were identified on the basis of growth on selective Rogosa SL agar (pH 5.2), cell morphology, gram staining, and catalase activity. Further identification of the species of these lactobacilli was performed according to carbohydrate fermentation patterns, esculin hydrolysis and growth at 15 ºC and 45 ºC in the Lactobacilli DeMan Rogosa Sharpe (MRS) broth (Difco), as described in Bergey's Manual of Systematic Bacteriology (6). The vaginal Lactobacilli strains isolated in this study were called TL (Türkiye Lactobacilli). Purified cultures were maintained at –80 ºC in MRS broth with 10% glycerol.

Preparation of culture supernatants: The bacteriocin-producing strains were grown in MRS broth (pH 5.5) at 37 ºC for 18-20 h. The lactobacilli culture was centrifuged at 10,000 rpm for 5 min, and then the supernatant was adjusted to pH 6.5-7.0 with 1N NaOH (7).

Bacteriocin assay: Bacteriocin activity was detected by the agar-spot test (7). The test was performed as follows: 200 µl of each *Lactobacillus* culture at the early exponential growth phase (OD600 of 0.2-0.3) in MRS broth was mixed with 4 ml of MRS soft agar (0.6% agar, prewarmed to 48 ºC) and poured on an MRS agar plate. Then, 3 µl of each culture supernatant was dropped onto the solidified soft agar. The plates were incubated for 48 h in a candle jar. Bacteriocin inhibition was indicated by a clear zone in the soft agar layer.

The antagonistic effects of culture supernatants of bacteriocin producing *Lactobacillus* strains on various gram positive, gram negative organisms and *C. albicans* were tested by the agar-well-diffusion assay as described by Toba et al. (8). In brief, 0.5 mm-diameter wells were made on preinoculated agar media and each well was filled with 100 µl of culture supernatant of bacteriocin-producing *Lactobacillus* strains after neutralization with NaOH. *G. vaginalis* was grown on Columbia agar (Difco), *C. albicans* was grown on Saboraud Dextrose agar (Becton Dickinson) and *S. aureus*, *S. milleri* IS51, *E. coli*, *E. cloacea* and *P. aeruginosa* were grown on Brain Heart Infusion agar (Difco). Aside from *G. vaginalis*, which was grown in the candle jar, all cultures were grown aerobically at 37 ºC for 48 h. Inhibition zones around the wells were measured and recorded.

Sensitivity to heat: To test heat sensitivity, 100 µl of culture supernatant was heated for 10 min at 60 ºC, 70 ºC, 80 ºC and 90 ºC. The agar spot test was performed to detect residual activity. The resistant culture supernatants were further heated for 10, 30 and 60 min at 100 ºC, and the residual activity was assayed (9).

Sensitivity to different pH values: The pH of culture supernatants was adjusted to 3.0, 4.5, 7.0 and 9.0 and then kept at room temperature for 4 h. Residual activity was determined by the agar-spot method as described (9).

Acid neutralization test: This test was performed by agar well diffusion assay (10). In addition to 100 µl of supernatants buffered with NaOH to 7.0, 75 µl of *Lactobacillus* suspension and 25 µl of 10% $CaCO_3$ solution were mixed and placed into the well. The original culture supernatants were used as control samples. When the inhibition zone was determined around the wells of both the control and buffered samples, the inhibitory effect was assumed to be due to bacteriocin or H_2O_2, and other tests were performed.

Sensitivity to catalase: Eighteen-hour cultures of strains showing antimicrobial activity were diluted at 1:10 in 10 mM Tris HCl (pH 7.0) and 2 µl of the suspension (about 10^6 cell/ml) was inoculated on Rogosa SL agar in tissue culture plate and incubated (10). Eight-hour growing cultures of indicator strains were diluted at a 1:10 ratio in 10 mM Tris HCl (pH 7.0) and mixed with

Rogosa SL soft agar (48 ºC). Catalase enzyme was then added at a final concentration of 0.5 mg/ml. The mixture was poured onto the tissue culture plate wells. One well having no catalase enzyme was used as the control. The tissue culture plates were examined after 18-24 h of incubation. The presence of an inhibition zone around wells both with and without catalase was determined to be the effect of bacteriocin.

Sensitivity to proteolytic enzymes and chloroform: Culture supernatants were treated with proteinase K, pronase E and trypsin, each at a final concentration of 0.1 mg/ml (11). The samples with and without proteases were incubated at 30 ºC for 1 h and residual activity was determined. To test for chloroform sensitivity, the culture supernatant was mixed with an equal volume of chloroform and kept at room temperature for 4 h before antimicrobial activity testing.

The effect of growth media on bacteriocin production: Two commercial media, Rogosa SL and MRS media, were tested for their ability to support the production of bacteriocin. Five milliliters of both broth media supplemented with 0.2 µg/ml mitomycin C^7 and without mitomycin C inoculated with bacteriocin-producing *Lactobacillus* strains and incubated at 37 ºC for 18 h. The bacteriocin activity of the culture supernatants was detected by agar-spot assay.

The effect of NaCl on bacteriocin production: After sterilization by autoclaving, MRS agars with (1%, 2%, 3% and 4% NaCl) and without NaCl were aseptically transferred onto a tissue culture plate. Bacteriocin-producing *Lactobacillus* strains (about 2 x 10^3 cells) in 2 µl were dropped onto the MRS agar and grown overnight. The fresh culture of the indicator strain was mixed with 0.6% MRS agar and spread onto the surface of the MRS agar containing the bacteriocin-producing *Lactobacillus* colonies followed by incubation for 24-48 h. Bacteriocin activity was assayed according to inhibition zones surrounding the colonies. The diameters of bacteria-free zones were measured and the differences were found by the Mann-Whitney U test.

Results

One hundred *Lactobacillus* strains were isolated from the vaginal samples of 75 reproductive women admitted to obstetrics and gynecology departments. The culture supernatants obtained from 100 lactobacilli isolates were tested for antibacterial activity against the same group of lactobacilli. Among them, six strains of lactobacilli were observed to have bacteriocin activity against eight of 10 different *Lactobacillus* species as well as *S. milleri*, *P. aeruginosa*, *E. coli*, *P. vulgaris*, *E. cloacea* and *G. vaginalis*. However, none of the six bacteriocins had inhibitory effects on *S. aureus* and *C. albicans* strains. All of the six bacteriocins showed inhibitory activity against *G. vaginalis* ATCC 14018 and *P. aeruginosa* ATCC 10145 among the sensitive bacteria tested (Table 1).

Bacteriocin sensitivity to physical conditions and chemical substances was also evaluated (Table 2). All six bacteriocins were completely destroyed by proteinase K, pronase E and trypsin treatment. However, the activity of six bacteriocins was maintained after catalase treatment, indicating that antibacterial activity was due to bacteriocin not H_2O_2.

Among the six bacteriocins, *L. gasseri* TL093c and TL143a strains were identified as lipid-containing bacteriocins because of their sensitivity to chloroform. These two bacteriocins were also inactivated by heating at 60 °C for 10 min. On the other hand, *L. gasseri* TL029, *L. delbrueckii* subsp. *delbrueckii* TL059a, *L. gasseri* TL080 and *L. acidophilus* TL099a were resistant to chloroform and resistant to boiling for at least 10 min. These bacteriocins belong to low-molecular weight non-lipid containing bacteriocins. All six bacteriocins were stable between pH 4.5 and 7.0, but sensitive to pH 9.0. Two bacteriocins isolated from *L. delbrueckii* subsp. *delbrueckii* TL059a and *L. acidophilus* TL099a were found to be active at pH 3.0 (Table 2).

The effects of media composition and mitomycin C on the production of bacteriocin were also evaluated. We found no significant difference between spontaneous induced MRS and Rogosa SL medians in terms of bacteriocin production (Mann-Whitney U test, $Z = -0.66$, $p > 0.05$). However, we found a significant difference between mitomycin C induced medians ($Z = -2$, $p < 0.05$). As seen in Table 3, Rogosa SL broth with mitomycin C had greater stimulatory effects particularly on the production of bacteriocins compared to those of MRS broth. Bacteriocin from *L. gasseri* TL029 was only produced after mitomycin C induction in both media. Among the six bacteriocins, bacteriocin produced by *L. delbrueckii* subsp. *delbrueckii* TL059a was not produced in Rogosa SL broth in the absence of mitomycin C.

Table 1. Effects of six bacteriocins on the growth of some bacteria on agar plates.

Indicator Strains	Bacteriocin-producing *Lactobacillus* strains					
	TL029 (L.g)	TL059a (L.dd)	TL080 (L.g)	TL093c (L.g)	TL099a (L.a)	TL143a (L.g)
L. gasseri	1*/44**	2/44	1/44	10/44	1/44	13/44
L. acidophilus	1/11	1/11	0/11	2/11	1/11	3/11
L. delbrueckii subsp. *delbrueckii*	0/14	0/14	0/14	1/14	0/14	1/14
L. delbrueckii subsp. *lactis*	0/9	0/9	0/9	1/9	0/9	1/9
L. fermentum	0/4	0/4	0/4	0/4	0/4	1/4
L. vaginalis	0/4	0/4	0/4	0/4	0/4	1/4
L. jensenii	0/2	0/2	0/2	1/2	0/2	1/2
L. coryniformis	0/1	0/1	0/1	1/1	0/1	1/1
L. agilis	0/1	0/1	0/1	0/1	0/1	0/1
L. casei	0/2	0/2	0/2	0/2	0/2	0/2
G. vaginalis ATCC 14018	1/1	1/1	1/1	1/1	1/1	1/1
E. coli ATCC 25922	0/1	1/1	0/1	0/1	0/1	0/1
P. vulgaris ATCC 13315	1/1	0/1	0/1	0/1	0/1	0/1
P. aeruginosa ATCC 10145	1/1	1/1	1/1	1/1	1/1	1/1
E. cloacea ATCC 13047	0/1	0/1	0/1	0/1	1/1	1/1
S. milleri IS51	0/1	0/1	1/1	0/1	0/1	0/1
S. aureus ATCC 25923	0/1	0/1	0/1	0/1	0/1	0/1
C. albicans ATCC 60193	0/1	0/1	0/1	0/1	0/1	0/1

* The number of sensitive TL and ATCC strains, ** The number of strains tested
L. g: *L. gasseri*, L. dd: *L. delbrueckii* subsp. *delbrueckii*, L.a: *L. acidophilus*

Table 2. Effect of catalase, protease, chloroform and heat treatment on bacteriocin activity.

Bacteriocin Producing *Lactobacillus* Strains	Sensitivity					Resistance to heating (10 min) Temperature (ºC)				Resistance to boiling (min)			Sensitivity Different pH values			
	P-K	P-E	Trp	Cat	Chl	60	70	80	90	10	30	60	3.0	4.5	7.0	9.0
TL029	R/S	S	S	R	R	R	R	R	R	R/S	S	S	S	R	R	S
TL059a	S	S	S	R	R	R	R	R	R	R	R	R	R	R	S	
TL080	S	S	S	R	R	R	R	R	R	R	R	R	S	R	R	S
TL093c	R/S	S	S	R	S	R/S	S	S	S	S	S	S	S	R	R	S
TL099a	R/S	S	R/S	R	R	R	R	R	R	R	S	S	R	R	R	S
TL143a	R/S	S	S	R	S	S	S	S	S	S	S	S	S	R	R	S

R= Resistance = Un inhibited bacteriocin activity, S= Sensitive = Inhibited bacteriocin activity
R/S= Moderate sensitivity, P-K= Proteinase K, P-E= Pronase E, Trp= Trypsin

Table 3. Inducible characteristics of bacteriocins in different growth media (mm).

Bacteriocin Producing Strains	Spontaneous Inducibility		Mitomycin C Inducibility	
	MRS	Rogosa-SL	MRS	Rogosa SL
L. gasseri TL029	0	0	1	1
L. d. subsp. *delbrueckii* TL059a	1	0	4	4
L. gasseri TL080	1	1	1	2
L. gasseri TL093c	0	3	2	4
L. acidophilus TL099a	2	2	2	3
L. gasseri TL143a	2	3	2	3

The effects of NaCl on the production of the six bacteriocins were studied. In both Rogosa SL and MRS broth, 1% NaCl increased the production of bacteriocins from 25 to 75% isolated from *Lactobacillus* strains of TL059a, TL080, TL093c and TL099a. Among them, bacteriocin from *L. gasseri* TL080 showed activity in the presence of 3% NaCl concentration, but this activity ended at 4% NaCl. Two bacteriocins from *L. casei* TL029 and *L. gasseri* 143a saw no increase in their activity, but were inhibited by more than 1% NaCl in both Rogosa SL and MRS media.

Discussion

Because viable lactobacilli can inhibit food-borne and enteric pathogenic microorganisms by producing lactic acid and other antimicrobial substances, yogurt and acidophilus milk have been considered to be healthy probiotic diets (4).

In this study, of 100 *Lactobacillus* culture supernatants examined for acid and bacteriocin production, six had inhibitory effects on sensitive bacteria including *Lactobacillus* strains of human origin and some common pathogenic bacteria colonized in the human intestine and vagina.

Among the bacteriocins tested, bacteriocins from *L. gasseri* TL93a and TL143a strains had a broader host range. We observed that all six bacteriocins had an inhibitory effect on *G. vaginalis* and *P. aeruginosa*. In addition, *L. casei* TL029 had an inhibitory effect on *P. vulgaris*, *L. delbrueckii* subsp. *delbrueckii* TL059a had an inhibitory effect on *E. coli,* and *L. acidophilus* TL099a, and *L. gasseri* TL143a had an inhibitory effect on *E. cloace.* However, none of them affected *S. milleri*, *S. aureus* or *C. albicans*.

An expanded host range has been noted recently for a number of *Lactobacillus* bacteriocins which kill *Enterococcus faecalis*, *Listeria monocytogenes, Clostridium botulinum, Candida tyrobutyricum*, *S. aureus* and *Aeromonas hydrophila* (1). Toba et al. determined bacteriocins in six *L. gasseri*, *L. acidophilus* JCM 1132 and *L. acidophilus* LAPT 1060 strains from infant feces active against other *Lactobacillus* stains (12). Kanatani et al. identified a bacteriocin (acidocin A) from *L. acidophilus* TK9201 (13). This bacteriocin had inhibitory effects on closely related lactic acid bacteria and food-borne pathogens including *Listeria monocytogenes*. Itoh et al. indicated that gassericin A produced by *L. gasseri* LA39 was one of the most active bacteriocins for use against enteric pathogens (14).

Silva et al. isolated a low-molecular weight substance from the *Lactobacillus* GC strain from the feces of a healthy person with potent inhibitory activity against a wide range of gram positive and negative bacteria (15). Coconnier et al. showed that the *L. acidophilus* LB strain was able to kill intracellular *S. thyphimurium* in the human intestinal Caco-2 cell culture model, and it decreased the colonization of bacteria in a mouse model due to its antibacterial activity (16).

In this study, six bacteriocins were characterized and tested for their in vitro antimicrobial activity against a group of microorganisms. All six bacteriocins were identified and their antimicrobial activity was differentiated from pH 3.0 to 9.0 and H_2O_2 by standard methods. Bacteriocin isolated from *L. gasseri* TL080 is considered to be a class IV bacteriocin with its resistance to chloroform and boiling. However, the other five bacteriocins shared several common features with other known *Lactobacillus* bacteriocins, and their classification deserves further study.

Bacteriocins characterized in this study were found to show antibacterial activity at a pH range of 4.0 to 7.0. In addition, two bacteriocins from *L. delbrueckii* subsp. *lactis* TL059a and *L. acidophilus* TL099a were active at as low as pH 3.0 and up to pH 7.0. Tagg et al. reported that most bacteriocins are resistant to acidic pH more than basic pH (12). The inhibitory activity of the bacteriocin isolated from *L. acidophilus* LB strain occurred between pH 3.0 and 5.0, and the inhibitory activity was lost when the pH was raised to 5.3 (17). Plantaricin S produced by *L. plantarum* LPC010 showed inhibitory activity from pH 3.0 to 7.0 (11).

To determine the effects of media composition on the production of bacteriocins, we used commercial Rogosa SL and Lactobacilli MRS media for culturing lactobacilli and in bacteriocin assays.

Sphelhaug et al. reported that MRS agar was suitable for bacteriocin assays of lactobacilli (18). We compared two media for their effects on bacteriocin production with or without mitomycin C, which has been shown to induce bacteriocin production. We found that Rogosa SL broth increased a bacteriocins of some *Lactobacillus* strains significantly compared to MRS broth in the absence of mitomycin. It was evident that Rogosa SL medium stimulated high-molecular weight and lipid-containing bacteriocin production more than MRS broth did. This effect may partly be due to the presence of high concentrations of tryptophane in Rogosa SL broth, which may constitute an important part of the proteinous part of such bacteriocin. Furthermore, we observed that 1% NaCl enhanced the bacteriocin production of six bacteriocins except for two bacteriocins isolated from *L. casei* TL029 and *L. gasseri* 143a strains. Larsen et al. detected bavaricin A from *L. bavaricus* M1401 (9). The production of this bacteriocin showed no changes at 1% NaCl, but production was inhibited with increaseing amounts of NaCl.

The natural inhibition of vaginal lactobacilli and some common pathogenic bacteria by bacteriocins may be important in understanding the initiation of vaginal infections or bacterial vaginosis associated with an unexplained decrease vaginal lactobacilli.

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