Antimicrobial Activity of Lactic Acid Bacteria Against *Listeria monocytogenes*

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ABSTRACT

Fourteen bacteriocin-producing strains from the genera *Lactobacillus, Leuconostoc, Pediococcus,* and *Lactococcus* were evaluated for their ability to inhibit the growth of eight strains of *Listeria monocytogenes*. Seven strains of lactic acid bacteria were antagonistic toward *L. monocytogenes* by deferred antagonism testing on agar. Cell-free supernatants from cultures of three of the seven bacteriocin-producing strains which inhibited growth of *L. monocytogenes* in deferred antagonism testing also inhibited growth in well diffusion assays. The eight strains of *L. monocytogenes* were identical in their sensitivity or resistance to bacteriocins. The action of the bacteriocins was eliminated by proteolytic enzymes.

Listeria monocytogenes was described originally by Murray et al. *(23)* as the causative agent of septicemia in rabbits. the bacterium has been isolated from a large number of environmental sources including vegetation, silage, wild birds, cattle, sheep, and raw milk *(8,10,12,33)*. Raw milk has long been implicated as a vehicle for transmission of human listeriosis *(16,19)*. Recently, cheese and pasteurized milk have also been implicated *(5,9)*. Although foods associated with human listeriosis are largely of animal origin, outbreaks have been documented in which raw cabbage *(29)* or raw celery, lettuce and tomatoes *(13)* were implicated as the source of *L. monocytogenes*.

The taxonomic classification of *L. monocytogenes* has been unclear, ranging from inclusion in the family *Corynebacteriaceae (3)* to "genera of uncertain affiliation" *(4)*. Currently, the bacterium is classified in Bergey's Manual of Systematic Bacteriology as a regular, nonspore-forming gram-positive rod *(30)*. In 1977, Wilkinson and Jones *(35)* conducted a taxonomic survey of *Listeria* and related bacteria and recommended the inclusion of *Listeria* in the family *Lactobacillaceae*. These relationships suggest the possibility that bacteriocin-like substances produced by lactic acid bacteria might inhibit *Listeria*. Recent reports have demonstrated inhibition of *L. monocytogenes* by bacteriocin-producing strains of *Pediococcus acidilactici (14,24)*. In the present study, eight *L. monocytogenes* strains were exposed to known bacteriocin-producing lactic acid bacteria of dairy, meat, and vegetable origin to determine if inhibition of this significant food-borne pathogen occurred.

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MATERIAL AND METHODS

The *L. monocytogenes* strains used as indicator organisms are detailed in Table 1. A nonpathogenic species, *Listeria innocua* ATCC 33090, was included for comparison. Bacteriocinogenic strains of lactic acid bacteria are given in Table 2. Two bacteriocinogenic strains, *Lactobacillus* sp. UAL11 and *Leuconostoc* sp. UAL14 were isolates from meat which produced proteinaceous inhibitors that displayed a bactericidal mode of action (Stiles and Harris, unpublished). *Lactobacillus* sp. C-136, a gram-positive, heterofermentative rod isolated from fermented pickle brine was also shown to produce a bacteriocin which inhibited several closely related species (Daeschel, unpublished). *P. acidilactici* PAC 1.0 *(11)*, kindly provided by C. Gonzales, produces the bacteriocin pediocin PA-1.

Listeria strains were subcultured weekly in Brain Heart Infusion broth (BHI, Difco Laboratories, Detroit, MI) at 37°C. Fresh, 18-24 h cultures were used as indicator organisms. Lactic acid bacteria were propagated in MRS broth (Difco), All Purpose Tween broth (APT, Difco), or modified MRS broth (BM) as specified by Wilkinson and Jones *(35)*. When agar media were required, 1.5% granulated agar (Difco) was added to the broth media.

Modifications of deferred antagonism *(31)* and well diffusion assays *(32)* were employed to evaluate inhibition of *Listeria* by lactic acid bacteria. Deferred antagonism experiments were conducted by spotting 1-3 ul of an overnight lactic acid bacterial culture onto the surface of a BHI agar plate and incubating at 30°C until good growth was evident (18-24 h). The plates were overlaid with 8 ml of BHI soft agar (0.75% agar) seeded with 8 ul of a *Listeria* culture. After overnight incubation at 37°C, the plates were examined for zones of inhibition in the *Listeria* cell lawn. Acid inhibition of *Listeria* did not occur using BHI agar with 0.2% glucose at an initial pH of 7.4. Experimental controls included: 1)

TABLE 1. *Strains of* Listeria *evaluated for sensitivity to bacteriocins produced by lactic acid bacteria.*

Species	Strain	Isolation source
L. monocytogenes	F-5069, serotype 4b	Raw milk
L. monocytogenes	Scott A, serotype 4b	Patient
L. monocytogenes	F-5027, serotype 1a	Raw milk
L. monocytogenes	ATCC 19115, serotype 4b	Patient
L. monocytogenes	675-3, serotype 1a	Raw milk
L. monocytogenes	DA-1, serotype 4	Jalisco cheese
L. monocytogenes	ATCC 15313, typestrain	Rabbit
L. monocytogenes	UAL501	Hospital origin
L. innocua	ATCC 33090, typestrain	Cow brain

addition of 1 mg/ml protease *(Streptomyces griseus* Type XIV protease, Sigma Chemical Co., St. Louis, MO) to the BHI agar to destroy any inhibitory activity ascribed to bacteriocins; and 2) use of an isogenic strain of *Pediococcus pentosaceus* FBB61-1, which does not produce pediocin A *(7)*, to insure that zones of inhibition were not the result of lactic acid production.

For well diffusion assays, cell-free supernatants of lactic acid bacteria were prepared from cultures propagated under growth conditions optimized for bacteriocin production *(17,22)*. Briefly, supernatants were neutralized with 1N NaOH to pH 6.5 and sterilized by filtration through a 0.22 µm membrane (low protein-binding, Millex-GV, Millpore, Bedford, MA), or by heating at either 60°C for 30 min or 100°C for 10 min, depending on the heat stability of the bacteriocin. Where appropriate, the supernatants were concentrated approximately 10 fold by dialysis against polyethylene glycol (PEG 8000, Sigma Chemical Co., St. Louis, MO) or by ultrafiltration using membranes with a 10,000 MW exclusion limit (Centricon Microconcentrator, Amicon Division, Danvers, MA). A 50-100 µl sample of the bacteriocin was placed in 5 mm diameter sealed wells which had been cut into a BHI agar plate. The supernatants were allowed to diffuse into the agar for 4 to 6 h at 20°C. BHI soft agar (8ml) was seeded with 8 µl of a *Listeria* culture and poured over the surface of the agar well plate. The plates were incubated aerobically at 37°C overnight and examined for zones of inhibition in the *Listeria* cell lawns. Protease-treated (1 mg/ml, 1 h, 37°C) bacteriocin preparations were used as negative controls. The efficacies of all bacteriocin-producing cultures and bacteriocin preparations thereof were demonstrated in control assays using sensitive indicator strains.

RESULTS AND DISCUSSION

Table 2 summarizes the results of this study. Of 14 bacteriocinogenic lactic acid bacteria tested, seven strains inhibited *L. monocytogenes* and *L. innocua* by the deferred antagonism assay. *Leuconostoc* sp. UAL14 was only inhibitory to *Listeria* on BHI agar when the glucose level was increased to 0.5% or greater. This inhibition was not attributed to increased acid production. Figure 1 illustrates the inhibitory activity of some of the lactic acid bacteria strains toward *L. monocytogenes* ATCC 15313. The inhibitory effect of all bacteriocins was drastically reduced by inclusion of 1 mg/ml protease in the agar medium.

In the well diffusion assay, cell-free supernatants from *Lactobacillus* sp. UAL11, *Leuconostoc* sp. UAL14, and *P. acidilactici* PAC 1.0 cultures inhibited *Listeria*. Although *P. pentosaceus* FBB61-1, *P. pentosaceus* L-7230, *Lactococcus lactis* SIK83, and *L. lactis* ATCC 11454 were inhibitory in deferred antagonism assays, these strains did not inhibit *Listeria* in well diffusion assays. In deferred antagonism on agar, producer colonies may either generate more bacteriocin or continuously excrete bacteriocin so as to replenish the inhibitor over the entire course of the antagonism test. When finite amounts of bacteriocin are present, as in well diffusion assays, *Listeria* cells in the indicator lawn may overcome the bacteriocin levels, either by proteolytic degradation of the inhibitor, or through the outgrowth of a mutant

TABLE 2. *Bacteriocinogenic strains of lactic acid bacteria and their inhibitory activity toward* Listeria *sp.*

Species	Bacteriocin	Source or Reference	Inhibition test	
			Deferred Antagonism	Well diffusion
Lactobacillus acidophilus C-7	Lactacin M	Muriana[a]	-	-
Lactobacillus acidophilus 88	Lactacin F	22	-	-
Lactobacillus acidophilus 11759	Lactacin B	2	-	-
Lactobacillus acidophilus ADH	Lactacin B	21	-	-
Lactobacillus helveticus 481	Helveticin J	17	-	-
Lactobacillus plantarum C-11	Plantaricin A	Daeschel[b]	-	-
Lactobacillus sp. C-136	Unnamed	This Study	-	-
Lactobacillus sp. UAL11	Unnamed	Shaw[c]	+	+
Lactococcus lactis ATCC 11454	Nisin	ATCC[d]	+	-
Lactococcus lactis SIK83	Nisin	1	+	-
Leuconostoc sp. UAL14	Unnamed	Shaw[c]	+	+
Pediococcus acidilactici PAC 1.0	Pediocin PA-1	11	+	+
Pediococcus pentosaceus FBB61-1	Pediocin A	7	+	-
Pediococcus pentosaceus FBB61-2[e]	None	7	-	-
Pediococcus pentosaceus L-7230	Pediocin A	7	+	-

[a]P. Muriana, M.S. thesis, N.C. State University, Raleigh, 1986.

[b]Daeschel, M.A., M.C. McKenney, and L.C. McDonald. 1986. Characterization of a bacteriocin from *Lactobacillus plantarum* C-11. Proceedings of the 86th Annual Meeting of the American Society for Microbiology, p. 133.

[c]B.G. Shaw and C.D. Harding, Institute of Food Research, Bristol, England.

[d]American Type Culture Collection, Rockville, MD.

[e]Cured of plasmid-borne bacteriocin determinants.

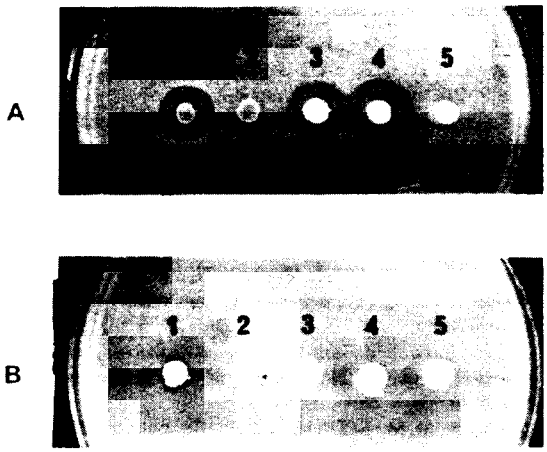

Figure 1. *Inhibitory activity of four bacteriocinogenic lactic acid bacteria toward* L. monocytogenes *ATCC 15313 by the deferred antagonism assay on BHI agar (A) and BHI agar with 1 mg/ml protease (B). 1* = Pediococcus pentosaceus *FBB61-1; 2* = P. pentosaceus *FBB61-2; 3* = Lactococcus lactis *ATCC 11454; 4* = L. lactis *SIK-83; 5* = Lactobacillus plantarum *C-11.*

population resistant to the bacteriocin. We did not evaluate these possibilities further.

All strains of *Listeria* reacted identically in terms of their general bacteriocin sensitivity or insensitivity, but the degree of sensitivity was variable among strains. This is in contrast to Hoover et al. *(14)*, who demonstrated inhibitory activity of *P. acidilactici* PO2 toward *L. monocytogenes* ATCC 19111, 19113, and 19115, but not *L. monocytogenes* F-5027 or F-5069 in deferred antagonism assays. These differences may be attributed to the inhibitory agent itself or to the agar medium utilized in the test.

This study has shown that antimicrobial proteins from select lactic acid bacteria are capable of inhibiting *L. monocytogenes*. Some of the lactic acid bacteria that have this ability have been shown to produce bacteriocins which inhibit bacteria across a wide range of gram-positive genera *(20)*. Examples of broadly active bacteriocins include nisin from *L. lactis (15)* and pediocin A from *P. pentosaceus* FBB61-1 *(7)*. It was not surprising that these bacteriocins were also active against *L. monocytogenes*. Lactic acid bacteria that produce bacteriocins which inhibit strains closely related to the producer organism (eg., helveticin J from *Lactobacillus helveticus* 481 *(17)* and lactacin F from *Lactobacillus acidophilus* 88 *(22)* did not inhibit *L. monocytogenes.*

With recognition of *L. monocytogenes* as a major foodborne pathogen, effective measures must be taken to minimize the contamination of food products. The pathogen has the ability to withstand a large variety of environmental conditions such as refrigeration temperatures *(34)*, salt concentrations as high as 10% *(30)*, and pH levels as low as 5.0 *(6)*. *L. monocytogenes* has also been shown to survive the processing and storage of fermented products such as hard salami *(18)*, cheeses *(25,26)*, and fermented milk *(27,28)* made from raw materials contaminated with the organism. These observations suggest that traditional food preservation processes may not prevent growth of *L. monocytogenes*. Modified approaches for preservation which will improve food safety and product shelf-life should be explored.

Some of the lactic acid bacteria used in this study are starter cultures for manufacture of fermented foods (cheese, yogurt, pickles, sauerkraut, and sausage). The ability of bacteriocin-producing lactic acid bacteria to inhibit pathogenic *L. monocytogenes* as well as other gram-positive food-borne pathogens provides an approach for control of these pathogens in fermented foods. Pucci et al. *(24)* were able to decrease viable counts and delay growth of *L. monocytogenes* in several refrigerated dairy products through the addition of a crude preparation of bacteriocin PA-1. Although PA-1 was more effective in an acidic food system, these results suggest that bacteriocins could be added exogenously to nonfermented foods to achieve acceptable levels of control. Use of bacteriocinogenic lactic acid bacteria as starter cultures or bacteriocins as food additives could be an effective approach to control *L. monocytogenes* in processed foods. A recent action by the Food and Drug Administration (FDA, Fed. Reg. 53:11247-11251, 1988), affirmed the GRAS status of nisin preparations for use in inhibiting *Clostridium botulinum* in cheese spreads. This decision provides a precedent for the use of bacteriocins to help ensure a safe food supply in the United States.

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