Complete Inhibition of Low Levels of *Listeria monocytogenes* on Refrigerated Chicken Meat with Pediocin AcH Bound to Heat-Killed *Pediococcus acidilactici* Cells†

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(Received 24 October 1995/Accepted 25 March 1996)

**ABSTRACT**

A pediocin-cell preparation from *Pediococcus acidilactici* H (pediocin AcH) was prepared by binding this bacteriocin to heat-killed producer cells by adjusting the pH of the medium to 6.0. This preparation, when added to irradiation-sterilized raw chicken breast meat in two experiments, had an inhibitory effect on *Listeria monocytogenes* Scott A during incubation at 5°C. In the first experiment, chicken with no pediocin-cell preparation (control) showed an increase of 1.9 log CFU/g, from 6.2 to 8.1 log CFU/g over 28 days from an initial challenge of about 5 log CFU/g of *L. monocytogenes* Scott A. A pediocin level of 2,400 AU/g decreased the *Listeria monocytogenes* Scott A count to 2.8 log CFU/g at 28 days. In the second experiment, at a challenge level of about 3 log CFU/g of *L. monocytogenes* Scott A, a pediocin addition of 2,400 AU/g caused a 1-log unit reduction at day 0, and from day 7 to 28 the counts were reduced to less than detectable levels. Conversely, counts in controls increased 1.9 log CFU/g, from 3.5 to 5.6 log CFU/g over 28 days. Although *L. monocytogenes* Scott A was inhibited, residual pediocin was not detectable in the exudates from treated raw chicken. Experiments with sodium dodecyl sulfate showed that the pediocin remained bound to chicken proteins and retained antilisterial activity. Conversely, pediocin did not bind as readily to cooked chicken. When bound to raw chicken before cooking, pediocin activity was found associated with the cooked chicken. Collectively, the data indicate that raw chicken treated with pediocin AcH exhibits antilisterial activity both before and after cooking, thus offering protection to consumers from foodborne illnesses caused by postprocessing recontamination and/or undercooking.

Key words: *Listeria monocytogenes*, pediocin, whole muscle chicken meat, *Pediococcus acidilactici*

*L. monocytogenes* (Lm) is a gram-positive, rod-shaped, psychrotrophic bacterium which was thought to be an animal pathogen until the 1970s (11). However, reported cases of human listeriosis increased rapidly in the 1980s. A potential factor contributing to concerns about food safety is the introduction and acceptance of microwave-oven technology. Precooked foods can be prepared in seconds by reheating; however, no bactericidal heat treatment is given prior to serving and *L. monocytogenes* can survive the short reheating. Therefore, foods contaminated with *L. monocytogenes* are not made safe when prepared in this way. The introduction of fully cooked, refrigerated, ready-to-eat poultry and other meat-based foods has raised concerns about *L. monocytogenes* control in cooked as well as raw poultry. *L. monocytogenes* has been isolated from raw chickens (1, 14, 21) and can be a potential source of contamination of chicken meat. A method for preventing *L. monocytogenes* growth on refrigerated raw or cooked meat would help reduce this potential hazard to public health.

Strains of *Pediococcus acidilactici* produce antimicrobial agents called pediocins, which inhibit gram-positive organisms (22, 23). *Pediococcus* spp. have been known for many years and occur naturally in or are added to fermented meats such as pepperoni and summer sausages. Pediocin AcH is a protein of about 3 kDa with bactericidal activity produced by *P. acidilactici* H (5, 6) that is inhibitory towards gram-positive bacteria.

Pediocins are also effective in preventing growth of *L. monocytogenes* in semidry and dry sausages (2, 12, 20), turkey sausage (23), wiener and wiener exudates (9, 10, 28), in fresh beef (20), in cottage cheese (21) and in various other foods (19, 26). The antibacterial properties of pediocins have been reviewed (15, 23, 25) and the nucleotide sequence coding for the protein determined (16, 18). Recently, pediocins and similar bacteriocins such as nisin were shown to exert their bactericidal activity against sensitive gram-positive pathogenic cells by disrupting and depleting the cellular proton motive force, thereby causing cell death (8, 17).

Pediocin AcH can be readsorbed by producer cells of *Pediococcus acidilactici* in spent culture broth media by raising the pH to 6.0 (3); this method has been used to partially concentrate and purify this bacteriocin (27). The
objectives of this study were to determine the effectiveness of such a reabsorbed natural pediocin preparation against growth of *L. monocytogenes* cells on raw and cooked chicken at refrigerator temperatures as a novel method of controlling or reducing this pathogen on foods.

**MATERIALS AND METHODS**

**Cultures**

Pure cultures of *L. monocytogenes* Scott A were grown on tryptase soy broth (TSB) (BBL, Cockeysville, MD) with 0.6% added yeast extract. Working cultures were inoculated from frozen stocks as needed and incubated at 37°C for 18 h. *Pediococcus acidilactici* H and the indicator organism *Lactobacillus plantarum* NCDO 995 were grown on lactobacilli MRS broth (MRS) (Difco Laboratories, Detroit, MI). Working cultures were inoculated from frozen stocks as needed and incubated at 30°C for 18 h.

**Preparation of sterile meat**

Fresh, whole, boneless chicken breast meat was purchased at a local supermarket. The meat was cut into 10.0 g pieces measuring approximately 3 by 3 by 1.5 cm. Pieces were packed flat in 0.47-liter resealable polyethylene plastic bags (Glad-Lock, First Brands Corp.) and frozen overnight at -70°C. Frozen packages were packed in dry ice, shipped by overnight express, and irradiated (30 kgy) in a linear accelerator unit at Iowa State University, Ames, IA. The sterile chicken pieces were confirmed to be free of vegetative bacterial cells by spread plating samples on TSA with 0.6% added yeast extract and incubating at 30°C for 48 h. Sterile chicken meat was stored at -70°C and was used within 30 days.

**Pediocin preparation**

Two liters of casein glucose broth (CGB) (6) was inoculated with 0.25% of an 18 h culture of *P. acidilactici* H and incubated at 30°C for 24 h with stirring on a Corning Stirrer/Hotplate Model PC-320 with a 5-cm stirring bar at speed 5. Cells were killed by heating to 80°C for 20 min. Free pediocin was bound to the killed cells by adjusting pH to 6.0 with 12 N NaOH and incubating for 30 min at 25°C with agitation (3, 7). Killed cells were harvested by centrifuging at 14,000 *X* g for 25 min. Cells were resuspended in a minimal volume of 5 mM phosphate-buffered saline (PBS) at pH 7.0. The suspensions were frozen at -70°C, lyophilized on a Trival B Freeze Dryer (The Virtis Corp., Gardiner, NY), designated as pediocin-cell preparation and stored at 4°C until used.

**Pediocin titer by bioassay**

One milligram of pediocin-cell preparation was rehydrated in 1 ml of 1 N gluconic acid lactone (Sigma Chemical Co., St. Louis, MO) and incubated for 30 min at 25°C on a reciprocal shaker (Labquake Model L-1237, Lab Industries, Inc., Berkeley, CA) at 6 cycles per min to free the pediocin from bacterial cells. The pH of the rehydrated pediocin-cell preparation was determined. The cell mixture was diluted serially; 5 μl of each dilution was tested against a lawn of *Lactobacillus plantarum* NCDO 955 containing about 3.5 × 10⁵ CFU/ml, and pediocin titer was determined as previously described (6).

**Pediocin titer by ELISA**

For titer measurements, 200 μl of rehydrated pediocin-cell preparation were placed in the first well of a 96-well microtiter plate (Immulon #2, Dynatech Laboratories, Inc., Chantilly, VA); 100 μl of 50 mM carbonate coating buffer, pH 9.6, was placed in the remaining wells of the plate. Pediocin-cell preparations were serially diluted and incubated at 4°C for 18 h. Plates were washed 5× with 20 mM PBS plus 0.5% Tween 20 (Sigma). Primary antibody MAb R2AR, which is specific for pediocins of *P. acidilactici* (4), was diluted 1:320 in 20 mM PBS and 100 μl were added to each well. The plates were incubated at 37°C for 1 h and washed as above. After addition of secondary antibody (Antimouse Polyvalent Ig, Sigma) at a dilution of 1:1,000, (100 μl/well) the color was developed by adding 100 μl of substrate solution per well. Substrate contained o-phenylenediamine (Sigma) at 1 mg/ml of 0.1 M sodium citrate buffer containing 0.4 ml of 30% H₂O₂/liters. Color development was stopped with 100 μl of 2 N H₂SO₄ per well. Plates were read on a BioRad 3550 Micro Plate Reader (BioRad, Richmond, CA) at 490 nm.

**Growth inhibition of *L. monocytogenes* by pediocin-cell preparation**

*Raw chicken at 5°C.* Six pieces of sterile chicken meat were used per plate. Four plates were used per trial and each trial was repeated three times. Chicken was thawed and one piece was placed in each well of a sterile 6-well Falcon tissue-culture plate (Becton-Dickinson, Lincoln Park, NJ). Treatments were as follows: well 1, control (meat only); well 2, 10⁶ CFU of *L. monocytogenes* per g of chicken; well 3, 2,400 AU of pediocin per g of chicken; wells 4, 5, and 6 received 10⁷ CFU of *L. monocytogenes* per g of chicken plus 800, 1,600, or 2,400 AU of pediocin per g of chicken, respectively. All wells received 4 ml of 5 mM PBS. Plates were prepared 4× for each replication and stored at 5°C. After incubation for 0, 7, 14, or 28 days, one plate was selected at random for analysis. The entire contents of each well were aseptically removed and placed in a separate filtering stomach bag (Tekmar, Cincinnati, OH) and each bag was stomached with a Stomacher Lab-Blender 400 (Tekmar) for 30 s. The filtered liquid was assayed for *L. monocytogenes* counts on spread plates containing TSA with 0.6% yeast extract on 150 by 15 mm Petri Plates (Fisher Scientific, Pittsburgh, PA) incubated at 37°C for 48 h. Pediocin activity was determined by bioassay and immunoassay (4).

Similarly, in a separate set of experiments 6 sterile raw chicken pieces were inoculated with about 10⁵ *L. monocytogenes* cells per g of chicken and the effects of the same amounts of pediocin on *L. monocytogenes* growth were studied as above.

*Cooked chicken at 8°C.* Six irradiated 10-g chicken breast pieces were aseptically transferred to separate sterile 4-½ oz Whirl Pak polyethylene sample bags (Nasco, Fort Atkinson, WI) and the bags were sealed. The bags were immersed in a water bath at 90°C for 20 min. The chicken was cooled after cooking by immediately immersing the bag in ice water. One piece of chicken was placed in each well of a 6-well tissue-culture plate with pediocin as above and inoculated with ca. 10⁶ CFU of *L. monocytogenes* per g of chicken. Plates were prepared in triplicate. Plates were incubated at 8°C for 20 days and the plate contents were assayed for *L. monocytogenes* counts and pediocin activity as described before.

**Binding study of pediocin to raw and cooked chicken**

Two cooked and two raw chicken pieces (10 g each) were placed in separate wells of a 6-well tissue-culture plate. Pediocin (2,400 AU per g of chicken) preparation in 4 ml of 5 mM PBS was added to each well. The plate was incubated at 5°C for 18 h. A 0.1-g sample was excised from each piece and placed on a prepared MRS agar plate containing a lawn of about 3.5 × 10⁶ CFU/ml of *L. plantarum*. The remaining chicken samples and well contents were then stomached for 30 s and 5 μl of tissue juice was spotted on the lawn. Protein-to-protein binding was broken by adding 1 ml of 10% sodium dodecyl sulfate (SDS) to each stomacher bag and
stomaching for 30 s. Bag contents were incubated for 30 min at ambient temperature. The bag contents were transferred to 1.5-ml microcentrifuge tubes (Fisher) and centrifuged at 10,000 × g for 20 min; 0.1 g of the pellet and 5 μl of the supernatant were spotted on the lawn plate. The plate was incubated at 30°C for 48 h.

**Effect of cooking on pediocin bound to raw chicken**

Five 10-g samples of chicken breast meat were placed in Whirl Pak bags and treated with 0, 800, 1,600, 2,400 or 3,200 AU of pediocin per g of chicken dissolved in 5 ml of 5 mM PBS. Samples were incubated at 5°C for 18 h. The chicken was cooked by immersing the bags in a 90°C water bath for 20 min. Bags and contents were chilled in ice water. The samples were placed in sterile 6-well tissue-culture plates and incubated for 7 days at 5°C. The chicken pieces were rinsed by immersion in sterile 5 mM PBS for 5 s to remove unbound pediocin and 0.1-g portions from each treatment were spotted on lawns of *L. plantarum*. The plates were incubated as above.

**Statistical analysis**

The data were analyzed using PROC ANOVA according to the General Linear Models procedures of SAS® Version 6.08 (24).

**RESULTS**

The inhibitory effects of pediocin-cell preparation on the growth of *L. monocytogenes* at 5°C on raw chicken breast are summarized in Table 1. Inoculated chicken not protected by pediocin showed increases in counts from 6.2 to 8.1 log CFU/g, i.e., an increase of about 2 log units over 28 days. Pediocin treatment at three different levels showed significant decreases in *L. monocytogenes* counts on day 0 which ranged from 1.8 to 2.6 log units. A pediocin concentration of 800 AU/g of chicken did not show any additional inhibition from day 7 to day 28, allowing a 1.5-log unit increase by day 28. A pediocin concentration of 1,600 AU/g held counts essentially level at 4.1 to 4.3 log CFU/g over 28 days. Conversely, a pediocin concentration of 2,400 AU/g of chicken further reduced the *L. monocytogenes* count from about 3.6 to 2.8 log CFU/g by day 28. Compared with the control, the 2,400 AU of pediocin per g treatment caused a difference of 5.3 log units at day 28.

The pediocin concentration in the wells decreased rapidly; pediocin was undetectable in well liquids after 14 days by either bioassay or ELISA (Table 1). The ELISA technique was more sensitive than the bioassay, being able to detect levels as low as 20 AU/g with MAb R2AR. Supplies of MAb R2AR were expended and ELISA analysis of pediocin concentration was not extended to later trials.

The action of pediocin was much more evident when low levels of *L. monocytogenes* (10^3 CFU/g) were inoculated (Table 2). A 2,400 AU/g level of pediocin reduced the *L. monocytogenes* count by 1 log unit at day 0 and to less than detectable limits (<1 CFU/g) at day 7 and beyond. Conversely, controls showed an increase in counts from 3.5 to 5.6 log CFU/g over 28 days at 5°C. Pediocin concentrations of 800 and 1,600 AU/g yielded 1.5-log unit decreases in counts, from about 3 log CFU/g to 1.5 and 1.3 log CFU/g, respectively.

Cooked chicken breast meat without pediocin and inoculated with *L. monocytogenes* at 5.4 log CFU/g had a final count after 20 days at 8°C of 10.7 log CFU/g. Pediocin at 800 AU/g slightly restricted the final count to 9.5 log CFU/g. Pediocin at 1,600 AU/g restricted the final count to 7.3 log CFU/g and 2,400 AU/g yielded a count of 6.9 log CFU/g of chicken when compared with the control. Compared to the control, there was a 3.8-log units lower count for the 2,400 AU/g treatment at 20 days (Figure 1).

These experiments showed that the pediocin-cell preparation at 2,400 or 1,600 AU/g of chicken substantially

**TABLE 1. Growth of Listeria monocytogenes from initial challenge of about 10^3 CFU/g and pediocin assay on irradiated raw chicken at 5°C**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lm (log CFU/g) Bioassay</th>
<th>Pediocin Bioassay</th>
<th>ELISA</th>
<th>Lm (log CFU/g) Bioassay</th>
<th>Pediocin (AU/g) Bioassay</th>
<th>ELISA</th>
<th>Lm (log CFU/g) Bioassay</th>
<th>Pediocin (AU/g) Bioassay</th>
<th>ELISA</th>
<th>Lm (log CFU/g) Bioassay</th>
<th>Pediocin (AU/g) Bioassay</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (PBS only)</td>
<td>0c b</td>
<td>0c</td>
<td>0d</td>
<td>0c</td>
<td>0a</td>
<td>0a</td>
<td>0c</td>
<td>0a</td>
<td>0a</td>
<td>0d</td>
<td>0a</td>
<td>0a</td>
</tr>
<tr>
<td>10^3 CFU/g Lm only</td>
<td>6.2a</td>
<td>0c</td>
<td>0d</td>
<td>6.5a</td>
<td>0a</td>
<td>0a</td>
<td>7.2a</td>
<td>0a</td>
<td>0a</td>
<td>8.1a</td>
<td>0a</td>
<td>0a</td>
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<tr>
<td>2,400 AU/g pediocin</td>
<td>0c</td>
<td>3,200a</td>
<td>2,560a</td>
<td>0c</td>
<td>0a</td>
<td>0a</td>
<td>0c</td>
<td>0a</td>
<td>0a</td>
<td>0d</td>
<td>0a</td>
<td>0a</td>
</tr>
<tr>
<td>10^3 CFU/g Lm + 800 AU/g pediocin</td>
<td>4.4b</td>
<td>200b</td>
<td>640c</td>
<td>4.7b</td>
<td>0a</td>
<td>0a</td>
<td>4.9b</td>
<td>0a</td>
<td>0a</td>
<td>5.9b</td>
<td>0a</td>
<td>0a</td>
</tr>
<tr>
<td>10^3 CFU/g Lm + 1,600 AU/g pediocin</td>
<td>4.1b</td>
<td>800b</td>
<td>1,280b</td>
<td>4.3b</td>
<td>0a</td>
<td>20a</td>
<td>4.6b</td>
<td>0a</td>
<td>0a</td>
<td>4.3c</td>
<td>0a</td>
<td>0a</td>
</tr>
<tr>
<td>10^3 CFU/g Lm + 2,400 AU/g pediocin</td>
<td>3.6b</td>
<td>3,200</td>
<td>2,560</td>
<td>3.5c</td>
<td>40a</td>
<td>4.5b</td>
<td>0a</td>
<td>0a</td>
<td>2.8d</td>
<td>0a</td>
<td>0a</td>
<td></td>
</tr>
</tbody>
</table>

Lm, *Listeria monocytogenes*.

b Mean of 3 replicates. Means with the same letter in a column are not significantly different (P > .05).
TABLE 2. Growth of Listeria monocytogenes from initial challenge of about 10^3 CFU/g and pediocin assay on irradiated raw chicken at 5°C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 0</th>
<th></th>
<th>Day 7</th>
<th></th>
<th>Day 14</th>
<th></th>
<th>Day 28</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Control (PBS only)</td>
<td>0c^b</td>
<td></td>
<td>0c</td>
<td></td>
<td>0c</td>
<td></td>
<td>0c</td>
<td></td>
</tr>
<tr>
<td>10^3 CFU/g Lm only</td>
<td>3.5 A</td>
<td></td>
<td>3.2 A</td>
<td></td>
<td>3.3 A</td>
<td></td>
<td>5.6 A</td>
<td></td>
</tr>
<tr>
<td>2,400 AU/g pediocin</td>
<td>0c</td>
<td>800 A</td>
<td>0c</td>
<td>400 A</td>
<td>0c</td>
<td>0 A</td>
<td>0c</td>
<td>0 A</td>
</tr>
<tr>
<td>10^3 CFU/g Lm + 800 AU/g pediocin</td>
<td>3.0 A</td>
<td>0 b</td>
<td>2.3 B</td>
<td>0 c</td>
<td>1.8 B</td>
<td>0 A</td>
<td>1.5 B</td>
<td>0 A</td>
</tr>
<tr>
<td>10^3 CFU/g Lm + 1,600 AU/g pediocin</td>
<td>2.8 A</td>
<td>800 A</td>
<td>2.0 B</td>
<td>200 B</td>
<td>1.7 B</td>
<td>0 A</td>
<td>1.3 B</td>
<td>0 A</td>
</tr>
<tr>
<td>10^3 CFU/g Lm + 2,400 AU/g pediocin</td>
<td>2.5 B</td>
<td>800 A</td>
<td>0 c</td>
<td>400 A</td>
<td>0 c</td>
<td>0 A</td>
<td>0 c</td>
<td>0 A</td>
</tr>
</tbody>
</table>

^a Lm, Listeria monocytogenes.

^b Mean of 3 replicates. Means with the same letter in a column are not significantly different (P > .05).

reduced the number of L. monocytogenes colony-forming units in both raw and cooked chicken meat over a 20 to 28 day period. The pediocin concentration was also monitored in the same chicken meat samples on a weekly basis by using bioassay and enzyme immunoassay. No pediocin was detected in the well liquids of chicken meat samples after 7 days (Table 1) or 14 days (Table 2) of storage. When 0.1-g portions of the raw whole muscle were placed on lawn plates, a zone of inhibition formed, indicating that the pediocin was bound to the raw chicken meat and remained biologically active. Conversely, cooked meat did not bind the pediocin, as shown in Figure 2, lane C. Pediocin activity remained with the well liquids and not with the chicken.

SDS treatment allowed bound pediocin to desorb from raw chicken, causing an increase in the apparent pediocin activity in the supernant (Fig. 2, Lane E). Subsequent work suggested that the best way to protect cooked chicken products against L. monocytogenes growth is to bind pediocin to the raw chicken before cooking. (Figure 3).

DISCUSSION

In two recent independent sausage fermentation studies (2, 11), the reductions in viable counts of added L. monocytogenes caused by pH decreases versus those caused by in situ pediocin production by Pediococcus cultures were...
PEDIOCIN CONTROLS LISTERIA MONOCYTOGENES ON CHICKEN MEAT

FIGURE 3. Lawn plate of Lactobacillus plantarum NCDO 955 on MRS medium with 0.1-g pieces of chicken. Raw pieces were treated with pediocin at four levels, then cooked at 90°C for 20 min. A, raw control; B–E, cooked pieces treated with pediocin: B, 800 AU/g; C, 1,600 AU/g; D, 2,400 AU/g; E, 3,200 AU/g of chicken.

compared. In both studies, sausages in which pediocin was produced showed 0.25 to 1.5 log units greater reductions of L. monocytogenes cell numbers than in sausages in which pediocin was not produced. The concentrations of pediocin achieved in these sausages were not reported. The addition of 100 AU of pediocin per ml in wiener exudates led to 3-log cycle reductions if added L. monocytogenes in 6 to 7 days at 25°C but only a 1-log unit reduction over 25 days for similar experiments run at 4°C (27). When L. monocytogenes cells were preadsorbed to sterile raw beef, the addition of 5,000 AU/ml of pediocin in crude supernatant preparations from P. acidilactici caused detachment of about 2 log units of high inocula (5 to 6 log CFU/g of meat) or moderate inocula (3 to 4 log CFU/g of meat) (20). When pediocin was added first, a 5,000 AU/ml level again caused 2 to 2.5 log units fewer L. monocytogenes to attach to pediocin-treated beef strips versus controls. Beef strips treated with 5,000 AU/ml of pediocin and stored refrigerated at 4°C for 7 to 21 days allowed about a 2-log unit lower attachment of a challenge inoculum of 5 to 6 log CFU of L. monocytogenes per g of meat. After 28 days of storage the reduction in attachment was only 1.5-log units lower (19).

Our goal was to treat poultry products with pediocin without producing acid products that would adversely affect product flavor. In cooked chicken, acid flavors from fermentation products are unacceptable. To separate the lacticidial effects of pH reduction from pediocin itself, we used a crude preparation of pediocin absorbed onto heat-killed producer cells (3). The bulk of the lactic acid produced during pediocin production was left in the discarded supernatant after centrifugation to collect heat-killed cells with bound pediocin. The pediocin preparation concentrated by producer-cell absorption caused no flavor problems in taste tests (data not shown) and could be incorporated into batter and/or breading operations or as a marinade ingredient.

When raw chicken was protected with 2,400 AU of pediocin per g, bactericidal reductions of 3.8 log units from an inoculum of 6.2 log CFU/g were observed. This reduction represents a substantially larger reduction of Listeria cells in

ACKNOWLEDGMENTS

This work was supported in part by grants from the Southeastern Poultry and Egg Association and the USDA Food Safety Consortium. The authors acknowledge the assistance of Marlene Janes and Joel Ferguson.

REFERENCES