INHIBITION OF *LISTERIA MONOCYTOGENES* BY *CARNOBACTERIUM PISCICOLA* IN VACUUM-PACKAGED COOKED CHICKEN AT REFRIGERATION TEMPERATURES

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ABSTRACT

Listeria monocytogenes *inhibition by bacteriocinogenic* Carnobacterium piscicola *DX or nonbacteriocinogenic* C. piscicola *2818 was examined in vacuum-packed minimally processed chicken cubes with gravy at 4, 8 and 15C.* C. piscicola *DX and* C. piscicola *2818 at* 10^4 *CFU/g were coinoculated individually with a pool of three* Listeria monocytogenes *strains at* 10^2 *CFU/g. At 4 and 8C,* C. piscicola *DX inhibited growth of* L. monocytogenes *by 3 logs, significantly* ($P<0.05$) *more than did* C. piscicola *2818. At 15C both* C. piscicola *strains inhibited* L. monocytogenes *by one log cycle. The pH of all inoculated systems decreased from an initial pH of 6.14 to final values ranging from 6.06 to 5.65 depending on the inocula used. Bacteriocin was detected in the systems coinoculated with* C. piscicola *DX. These studies demonstrate that lower temperatures and bacteriocin production enhanced* L. monocytogenes *inhibition by* C. piscicola.

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INTRODUCTION

Interest in the development of minimally processed meat products has increased in recent years. These foods often receive only a mild heat-treatment, are free of additives, and rely on vacuum-packaging and refrigeration for their preservation. These barriers may not be sufficient to prevent the growth of psychrotrophic foodborne pathogens (Schofield 1992; Gould 1996), particularly *Listeria monocytogenes.* This organism poses a health threat, grows at 4C and will multiply faster in the event of any temperature abuse (Montlagh *et al.*1992). *L. monocytogenes* commonly occurs on raw meat and chicken (Pini and Gilbert 1988; Bailey *et al.*1989; Muriana 1996), and has also been isolated from ready-to-eat products (Harrison and Carpenter 1989).

Many bacteriocins produced by lactic acid bacteria inhibit *L. monocytogenes* (Lewus *et al.* 1991; Winkowski *et al.*1993; Goff *et al.*1996; Muriana 1996). However, the amount of acid produced may render the product "spoiled". Thus, *Carnobacterium*, which produce less acid, may be superior for biopreservation applications. Several *Carnobacterium spp.* have antilisterial bacteriocinogenic activity (Buchanan and Klawitter 1992; Mathieu *et al.* 1994). *C. piscicola* DX, a strain isolated in our laboratory from refrigerated meat, also produces a bacteriocin that inhibits *L. monocytogenes* (Lewus *et al.*1991).

This study examined the inhibitory activity of *C. piscicola* against *L. monocytogenes* in vacuum-packaged minimally processed chicken cubes with gravy. We report here that *C. piscicola* inhibited *L. monocytogenes*, with a bacteriocinogenic strain being more inhibitory than a nonbacteriocinogenic strain. The inhibition depended on the storage temperature.

MATERIALS AND METHODS

Bacterial Strains and Conditions

L. monocytogenes Scott A, F 5069, and ATCC 19115, all carrying the plasmid pGK12 coding for chloramphenicol and erythromycin resistance (Foegeding *et al.*1992), were the gift of P.M. Foegeding (North Carolina State University, Raleigh). The presence of this plasmid allowed a medium containing chloramphenicol and erythromycin to be used for the selective enumeration of these strains (see below). *L. monocytogenes* cultures were grown at 30C in Trypticase soy broth without dextrose (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 0.6% yeast extract (Difco Laboratories, Detroit, Mich.), 0.5% dextrose, (Fisher Scientific Company, Pittsburgh, Pa.), 3.5 μg/mL chloramphenicol (Sigma Chemical Co., St. Louis, Mo.), and 3.5 μg/mL erythromycin (Sigma) (TSBYGA). *C. piscicola* DX which was isolated in our laboratory (Lewus *et al.*1991) and *C. piscicola* 2818, a bacteriocin nonproducer

(Bac$^-$) which was a gift of R. Buchanan (U.S Department of Agriculture, Wyndmoor, PA) were grown in Lactobacilli MRS broth (Difco) at 30C. The cultures were maintained as slants on MRS agar, kept at 4C and transferred monthly.

Inoculum Preparation

Overnight cultures of *C. piscicola* DX and *C. piscicola* 2818 were centrifuged (5000 × *g* for 20 min at 4C). The pellet was washed twice with, and resuspended in 0.1% peptone water. The cell suspensions were brought to the same absorbance (660 nm) by diluting with 0.1% peptone water to obtain the desired inoculum level. The three *L. monocytogenes* cell suspensions were prepared in a similar fashion and combined to use as a pool. In this study, *L. monocytogenes* strains were used as a pool in order to diminish the chance of selecting a strain very sensitive or insensitive to the bacteriocin. Furthermore, the sensitivity towards bacteriocin activity of *L. monocytogenes* strains transformed with the pGK12 plasmid, which conferred resistance to chloramphenicol and erythromycin, is the same as the wild type strains (Foegeding *et al.* 1992). The pool of *L. monocytogenes* Scott A, F 5069, and ATCC 19115 will be referred as to *L. monocytogenes.*

Chicken and Gravy Preparation

Fresh, refrigerated, skinless boneless chicken breast was purchased at a local supermarket. The superficial fat was removed and the chicken was cut into pieces (~1 cm^3), weighed in 90 ± 1g portions into gas impermeable bags, vacuum-sealed and steamed for 10 min at 100C. Heat treated bags were stored for one day at 4C before inoculation.

The gravy was prepared using commercially available canned chicken broth supplemented with 1.8% Trypticase peptone (BBL), 0.2% carrageenan type II (Sigma) and 2% corn starch (CPC International Inc., Englewood Cliffs, NJ) and autoclaved. Aliquots of 10 mL of gravy containing the inoculum were added aseptically using a syringe through a septum in the chicken bags giving a final inoculum level of 10^4 CFU/g of *C. piscicola* DX or *C. piscicola* 2818 and 10^2 CFU/g of *L. monocytogenes.* Uninoculated samples and each strain inoculated alone were used as controls. After inoculation, the chicken cubes with gravy were mixed throughout the samples by massaging the bags and then stored at 4, 8 and 15C for up to 28, 18 and 14 days, respectively.

Sampling and Analysis

Three bags from each treatment were sampled at selected times to determine *L. monocytogenes*, *C. piscicola* DX and *C. piscicola* 2818 populations and the pH. Each bag was placed in a stomacher bag, cut open, and 100 mL of peptone water were added. The samples were blended for 1 min using a stomacher (Model 400,

Dynatech Laboratories, Inc., Alexandria, VA). *L. monocytogenes* was selectively enumerated by pour plating or surface plating on TSBGYA agar (containing chloramphenicol and erythromycin) and incubating at 30C for 48 h. The indigenous biota could not grow on this medium (data not shown). The total aerobic population was enumerated on Plate Count Agar (PCA) (Difco). Since PCA allows the growth of *L. monocytogenes* and the indigenous flora, the difference in count between PCA and TSBGYA was used to determine the level of indigenous flora. *C. piscicola* DX and *C. piscicola* 2818 populations were determined by surface plating on MRS, using a spiral plater (Spiral Systems Instruments, Inc.). MRS does support the growth of *L. monocytogenes*, but since these populations were present at levels at least 100-fold lower than the *Carnobacteria,* they were diluted out and did not appear on the "countable" plates. Colonies were enumerated after 48 h at 30C with a bacterial colony counter (model 500A, Spiral Systems Instruments, Inc.). The pH of each sample was determined by inserting a flat-surface electrode into an aliquot of 0.5 mL of gravy.

Agar Diffusion Assay for Bacteriocin Production

Bacteriocin production was determined with the well diffusion agar assay (Rogers and Montville 1991) using the pool of *L. monocytogenes* strains as indicator. Equal cell densities of each strain were combined and diluted to 10^5-10^6 CFU/mL in tempered TSBGYA agar prepared using 2-(N-morpholino)-ethanesulfonate buffer (100 mM MES buffer pH 6.5) to neutralize acid production and supplemented with 0.1% of Tween 20 (Sigma) and 1.0% of Noble agar (Difco). The inoculated agar (20 mL) was pipetted into sterile petri dishes and 6.8 mm wells were cut after the agar solidified. Gravy samples (1 mL) at each sampling time were centrifuged in a microcentrifuge for 10 min. Fifty microliters of the cell-free supernatant were pipetted into the wells. The plates were incubated at 30C for 24 h. The clear zones formed around the wells were measured as the distance from the edge of the well to the end of the inhibition zone. To determine the equivalent nisin International Units (IU) per mL, the inhibition zones were compared to those of known nisin (Sigma) concentrations as described by Rogers and Montville (1991).

The proteinaceous nature of the inhibitory substance was confirmed by adding10 μL of α chymotrypsin (bovine pancrease, type II) (Sigma), 10 mg/mL in 10 mM sodium phosphate buffer (pH 7.5), into a 3.8 mm well cut adjacent to the 6.8 mm well (Lewus *et al.*1991). A negation of the inhibition zone around the protease indicated that the inhibitor was sensitive to the protease and thus confirmed that the inhibition was caused by a bacteriocin.

Statistical Analysis

An analysis of variance (ANOVA) and the Tukey test were performed to determine significant differences among log bacterial populations, pH values and bacteriocin production. In all cases significance is expressed at a 5% level.

RESULTS AND DISCUSSION

Growth of *L. monocytogenes* alone and growth of *L. monocytogenes, C. piscicola* DX, and *C. piscicola* 2818 from the coinoculated systems in vacuum-packed minimally processed chicken cubes with gravy at 4C are shown in Fig. 1, at 8C in Fig. 2, and at 15C in Fig. 3. The number of colonies formed on PCA was higher than that obtained using TSBGYA, due to the presence of an indigenous biota. This biota did not prevent *L. monocytogenes* from reaching levels of 10^7-10^8 CFU/g after 14 days at 4C (Fig. 1), 12 days at 8C (Fig. 2) and 4 days at 15C (Fig. 3), when inoculated alone at 10^2 CFU/g.

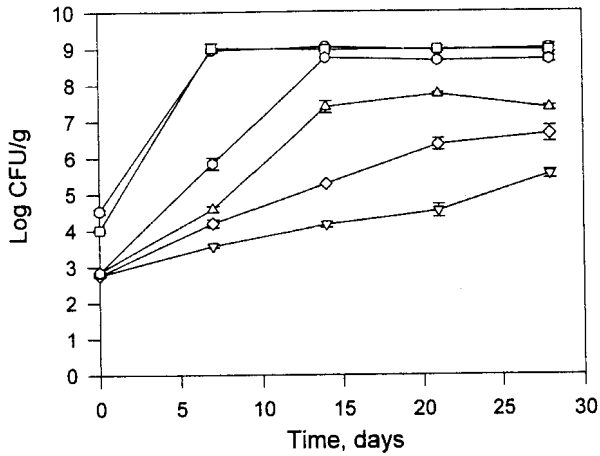

FIG. 1. GROWTH OF *L. MONOCYTOGENES, C. PISCICOLA* DX AND *C. PISCICOLA* 2818 IN VACUUM-PACKED MINIMALLY PROCESSED CHICKEN CUBES WITH GRAVY AT 4C
Symbols: ○ *C. piscicola* DX coinoculated with *L. monocytogenes* enumerated on MRS; □ *C. piscicola* 2818 coinoculated with *L. monocytogenes* enumerated on MRS; ∇ *L. monocytogenes* coinoculated with *C. piscicola* DX enumerated on TSBGYA; ◊ *L. monocytogenes* coinoculated with *C. piscicola* 2818 enumerated on TSBGYA; Δ *L. monocytogenes* alone enumerated on TSBGY;⬭ *L. monocytogenes* alone enumerated by total plate counts. Vertical bars represent standard deviation of the mean.

C. piscicola DX and *C. piscicola* 2818 populations reached levels of approximately 10^8-10^9 CFU/g during the first week of storage (Fig. 1, 2 and 3). The two strains grew similarly and were not influenced by the incubation temperature

nor the presence of *L. monocytogenes* (growth of *C. piscicola* DX and *C. piscicola* 2818 alone is not shown). At 4 and 8C, growth of *C. piscicola* DX inhibited *L. monocytogenes* by 3 log cycles during the second to the third week of storage. At 4 and 8C, *C. piscicola* 2818 also inhibited *L. monocytogenes*, but to a significantly ($p<0.05$) lesser extent (Fig. 1 and 2). A similar difference in inhibition between a bacteriocin producer and nonbacteriocin producer strains was also reported for pediococcal starter cultures (Berry *et al.* 1990; Foegeding *et al.* 1992; Luchansky *et al.* 1992). No significant differences between the inhibition against *L. monocytogenes* by *C. piscicola* DX and *C. piscicola* 2818 were observed at 15C. In both systems, one log reduction was observed throughout the storage period (Fig. 3).

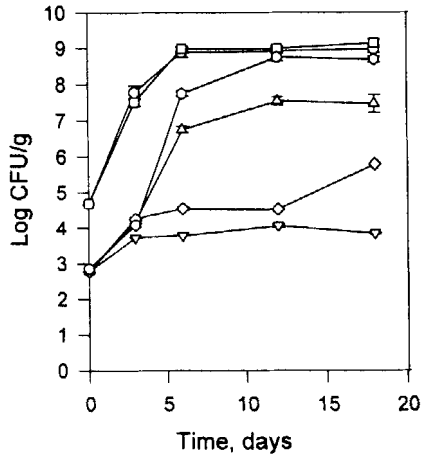

FIG. 2. GROWTH OF *L. MONOCYTOGENES, C. PISCICOLA* DX AND *C. PISCICOLA* 2818 IN VACUUM-PACKED MINIMALLY PROCESSED CHICKEN CUBES WITH GRAVY AT 8C
Symbols: see Fig. 1.

The pH values of the initial chicken gravy system was 6.14 ± 0.05. At the end of the incubation period pH values ranged from 5.65 to 6.06 (Table 1). Slight decreases in pH values were probably caused by the fermentation of dextrose contained in the chicken broth. Only at 4C, did coinoculation of *C. piscicola* strains produce a statistically significant reduction of the final pH relative to *L. monocytogenes* inoculated alone. *L. monocytogenes* can grow at pH 5.0 (George *et al.* 1988; Mathieu *et al.* 1994), therefore, the inhibitory action cannot solely be attributed to the small decrease in pH observed here.

All cell-free supernatants from samples coinoculated with *C. piscicola* DX produced an inhibition zone in the well diffusion assay. A negation of the inhibition zone in the presence of a protease demonstrated the proteinaceous nature

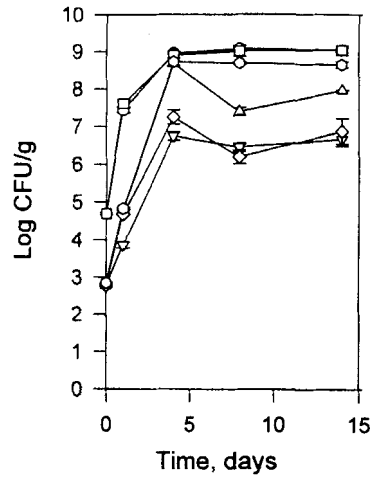

FIG. 3. GROWTH OF *L. MONOCYTOGENES*, *C. PISCICOLA* DX AND *C. PISCICOLA* 2818 IN VACUUM-PACKED MINIMALLY PROCESSED CHICKEN CUBES WITH GRAVY AT 15C
Symbols: see Fig. 1.

TABLE 1.
FINAL pH VALUES OF THE CHICKEN GRAVY SYSTEM INOCULATED WITH *C. PISCICOLA* AND/OR *L. MONOCYTOGENES*[a]

Inoculum (CFU/g)[b]			Final pH (±SD) at:		
DX	2818	*L. m*	4C	8C	15C
0	0	0	6.17 ± 0.08	5. 99 ± 0.06	6.10 ± 0.04
10^4	0	0	5.83 ± 0.05	5.65 ± 0.10	5.65 ± 0.02
0	10^4	0	5.82 ± 0.02	5.72 ± 0.05	5.69 ± 0.07
10^4	0	10^2	5.85 ± 0.11	5.74 ± 0.05	5.75 ± 0.03
0	10^4	10^2	5.88 ± 0.03	5.77 ± 0.05	5.80 ± 0.10
0	0	10^2	6.06 ± 0.07	5.82 ± 0.11	5.79 ± 0.09

[a] pH values measured after 28 days at 4C, 18 days at 8C and 14 days at 15C. The initial pH value for the three temperatures was 6.14 ± 0.05.
[b] DX, *C. piscicola* DX; 2818, *C. piscicola* 2818 and *L. m, L. monocytogenes.*

of the inhibitor. The inhibition zones measured at several time points during the incubation period at a given temperature were similar. These zone sizes were averaged (triplicates) to give the values shown in Table 2. Supernatants from uninoculated samples or from samples inoculated with the Bac$^-$ strain did not produce any inhibition zone in the well diffusion assay, suggesting that neither *C.*

piscicola 2818 nor the indigenous biota produced bacteriocins active against *L. monocytogenes*. The coinoculated system worked better at the lower temperature despite the fact that the bacteriocin level significantly ($p<0.05$) increased with increasing temperature (Table 2). Moreover, at 15C similar inhibition was caused by either *C. piscicola* strain. Thus, the decrease in *L. monocytogenes* growth could be attributed to some other factor produced by both *Carnobacterium piscicola* strains other than the bacteriocin or by *L. monocytogenes* cells being more sensitive to the bacteriocin at lower temperatures. Factors like low temperature, low NaCl concentrations, and pH values just below neutral were also reported to enhance *L. monocytogenes* inhibition by *C. piscicola* in synthetic media (Buchanan and Bagi 1997). Since the coinoculation of *L. monocytogenes* and *C. piscicola* results in competition for growth limiting compounds (Buchanan and Bagi 1997), initial inocula levels of both cultures may determine the suppression of the pathogen by the lactic acid bacteria.

TABLE 2.
BACTERIOCIN PRODUCTION IN THE CHICKEN GRAVY SYSTEM COINOCULATED WITH *L. MONOCYTOGENES* AND *C. PISCICOLA*[a]

	Level of bacteriocin produced[a] at:		
Coinoculation with:	4C	8C	15C
C. piscicola DX	89.7 ± 5.1	116.0 ± 8.0	135.7 ± 7.6
C. piscicola 2818	0	0	0

[a] Values represent mean ± standard deviation of bacteriocin production by *C. piscicola* expressed as nisin equivalent units (IU/mL).

C. piscicola DX was more effective at refrigeration temperature, suggesting a cooperative action between low temperature and bacteriocin activity. *C. piscicola* CP5 and *C. piscicola* LK5 (Buchanan and Klawitter 1992; Mathieu *et al.* 1994) and *L. sake* MN (Winkowski *et al.* 1993) also showed an enhanced inhibitory activity against *L. monocytogenes* at lower temperatures.

The *L. monocytogenes* population increased during 4 weeks of storage at 4C (Fig. 1). This growth might be attributed to either partial bacteriocin inactivation or the emergence of bacteriocin-resistant *L. monocytogenes* (Foegeding *et al. 1992*; Mathieu *et al.* 1994). To test the latter, *L. monocytogenes* isolates which had grown in the coinoculated systems at the end of the storage period were assayed for sensitivity towards the bacteriocin by the agar diffusion assay. Since the inhibition zones were similar to those of the original *L. monocytogenes* strains (data not shown), resistance to the bacteriocin was ruled out.

In this study, we demonstrated the antagonistic action of *C. piscicola* DX against *L. monocytogenes* and that this action is enhanced by the presence of

bacteriocin and by low temperature. Clearly *C. piscicola* DX can contribute to the biopreservation of refrigerated foods against *L. monocytogenes* although it is ineffective at abuse temperatures.

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