

# Microbial Competition: Effect of Culture Conditions on the Suppression of *Listeria monocytogenes* Scott A by *Carnobacterium piscicola*<sup>†</sup>

ROBERT L. BUCHANAN\* and LORI K. BAGI

## ABSTRACT

The effects of temperature (4, 12, and 19°C), pH (5, 6, and 7), and NaCl (5, 25, and 45 g/liter) on the growth of *Listeria monocytogenes* Scott A in the presence of either *Carnobacterium piscicola* LK5 or 2762 were studied quantitatively in brain heart infusion broth. Strain LK5 produces a bacteriocin that is released into the environment, whereas 2762 appears to produce a bacteriocin that remains cell associated. The primary effect of both *C. piscicola* strains was a suppression of the maximum population density (MPD) attained by *L. monocytogenes*. The extent of this depression was dependent on the three culture variables, and appeared to be a function of their influence on the relative growth rates of the two species. The effects were similar with both strains. However, two bacteriocin-negative strains, 2305 and 2818, also depressed the growth of *L. monocytogenes*. Little of the *C. piscicola* isolates' ability to suppress *L. monocytogenes* appeared attributable to bacteriocin production. The MPD-depressing activity of 2762 could not be attributed to peroxide, pH depression, or oxygen depletion. However, MPD suppression may involve nutrient depletion, since the extent of MPD suppression was decreased in a dose-related manner when the two species were cultured in 3× and 6× brain heart infusion broth.

Key words: Bacteriocins, pH, temperature, sodium chloride, modeling

Immediately after slaughter or capture, the microbiota of raw meat, poultry, and seafoods is diverse. However, as the animal tissues are chilled, packaged, and stored, a limited number of characteristic bacterial species predominate. These are the species best adapted to the environment that the food product presents. The growth of this characteristic microbiota is often cited as one of the factors that may influence the growth of foodborne pathogens in food products; however, there are few studies that have actively tested

this supposition, particularly in a quantitative manner. Interest in the role of microbial competition in assuring food safety has increased as a variety of bacteriocin-producing strains of bacteria occurring commonly in foods have been identified and are being evaluated as potential means for controlling foodborne pathogens. This is of particular interest with psychrotrophic species such as *Listeria monocytogenes*, where additional hurdles are needed to prevent growth in refrigerated products.

The interaction between *L. monocytogenes* and *Carnobacterium piscicola*, and how this is influenced by environmental conditions, was selected as the initial phase of a project attempting to quantitatively assess and ultimately model the impact of microbial competition on the growth of foodborne pathogens. *Carnobacterium* is a recently recognized genus that previously had been grouped among the "atypical lactic acid bacteria." Members of this genus are readily isolated from a variety of raw meat, seafood, and poultry products. There has been increased interest in *Carnobacterium* in the past several years because many isolates produce bacteriocins that are active against *L. monocytogenes* (1, 2, 6–11). Buchanan and Klawitter (2, 3) demonstrated that the antilisterial activity of *C. piscicola* LK5 was strongly dependent on temperature, with maximal activity associated with refrigeration temperatures. The objective of the current study was to quantitatively evaluate the effects that temperature, pH, and sodium chloride have on the ability of *L. monocytogenes* to grow in the presence of either *C. piscicola* LK5 or 2762. The former strain produces a bacteriocin that is excreted into the environment while the latter produces a bacteriocin that appears to remain associated with the cell. Additional experimentation was also conducted to more fully explain the mechanism(s) by which *C. piscicola* 2762 inhibits *L. monocytogenes* when the two microorganisms are cultured together.

## MATERIALS AND METHODS

### Microorganisms

*Listeria monocytogenes* Scott A and *Carnobacterium piscicola* LK5 were obtained from the laboratory's stock culture

collection. Scott A was originally isolated from a clinical sample and was acquired initially from the U.S. Food and Drug Administration. LK5 was isolated from raw ground beef (2). *Carnobacterium piscicola* 2762, 2818, and 2305 were acquired from the AFRC Institute for Food Research laboratory in Reading, England. These strains were originally isolated from fish, human, and irradiated chicken, respectively. Working stock cultures of *C. piscicola* strains were maintained in brain heart infusion broth (BHI) (Difco Laboratories, Inc., Detroit, MI) incubated at 28°C for 18 to 24 h and then stored at 4°C. Working stock cultures were subcultured monthly. *L. monocytogenes* was handled in a similar manner except that it was incubated at 37°C.

Prior to each experiment, a loopful of a working stock culture was transferred to a 250-ml Erlenmeyer flask containing 50 ml of BHI and cultured for 24 h on a rotary shaker (150 rpm). *C. piscicola* and *L. monocytogenes* were cultured at 28 and 37°C, respectively.

#### Co-culture experiments

Co-cultures were prepared and sampled using the techniques described by Buchanan and Klawitter (2). BHI was prepared, additional sodium chloride added if necessary to achieve target levels of 5, 25, or 45 g/liter, the pH adjusted to 5.0, 6.0, or 7.0 using concentrated HCl, and the broth brought up to volume. The broth was then transferred in 100-ml portions to 250-ml Erlenmeyer flasks, which were sealed with foam plugs, and sterilized by autoclaving for 20 min at 121°C. The pH of the medium after autoclaving remained within  $\pm 0.1$  pH units of the original value.

The flasks were inoculated with 0.1 ml of appropriate dilutions of the 24-h cultures of *L. monocytogenes* and either *C. piscicola* LK5 or 2762 to achieve target inoculum levels of approximately  $10^3$  CFU/ml. Flasks inoculated with only *L. monocytogenes* were used as controls. The flasks were then incubated on rotary shakers (150 rpm) at 4, 12, or 19°C.

Periodically, 4.0-ml samples of each co-culture and each control culture were removed, diluted appropriately in sterile 0.1% peptone water, and surface plated in duplicate on BHI agar (BHIA) (Difco) and Vogel Johnson agar (VJA) (Difco) using a Spiral Plater (Model D, Spiral Systems, Bethesda, MD). The BHIA plates were incubated for 24 h at 28°C and the VJA plates were incubated for 24 h at 37°C. If the *L. monocytogenes* colonies on VJA were small, the plates were incubated for an additional 24 h. The plates were counted using a Spiral Systems Laser Colony Counter (Spiral Systems) and converted to log values. The BHIA counts reflected the total count of both *C. piscicola* and *L. monocytogenes* colonies, whereas VJA only supported the growth of *L. monocytogenes*. The counts obtained for the *L. monocytogenes* controls on BHIA (data not shown) and VJA were virtually identical. In those instances where *C. piscicola* grew more rapidly in co-culture than *L. monocytogenes*, the number of colonies of the lactic acid bacteria could be estimated by the BHIA count.

#### Curve fitting

Growth curves were generated by fitting the data to the Gompertz equation as previously described (4, 5). The Gompertz parameters were then used to calculate lag phase duration (LPD), exponential growth rate (EGR), generation time (GT), and maximum population density (MPD).

#### Characterization of antilisterial activity

The *Carnobacterium* strains were screened for bacteriocin production using the spot-on-the-lawn antagonism test (14). *C. piscicola* 2762 was evaluated for cell-associated antilisterial activity by washing cells cultured in BHI at 28°C for 18 to 24 h with 5 mM sodium phosphate buffer (pH 6.5) and resuspending them in

100 mM NaCl (pH 2.0). This suspension was held on ice at 4°C for 1 h. The suspension was then centrifuged (20 min at  $22,000 \times g$ ) and the cells resuspended in phosphate buffer (pH 6.5). The supernatant and cells were then individually tested for listericidal activity using a well-diffusion assay (13). No inhibition of *L. monocytogenes* was noted when 100 mM NaCl (pH 2.0) without *C. piscicola* 2762 cells was assayed.

The sensitivity of the antilisterial activity of *C. piscicola* 2762 to three proteases was assessed using well-diffusion assays. Cell-free supernatants and washed cells were prepared from 50-ml overnight cultures of 2762 incubated with agitation at 28°C. After being harvested by centrifugation, the cells were washed three times and resuspended in 2 ml of 5 mM phosphate buffer (pH 6.5). The cell-free supernatant was filter-sterilized using a nylon filter (0.45- $\mu$ m pore size) that did not retain the bacteriocin. The resuspended cells or cell-free supernatant were transferred in 250- $\mu$ l portions to microcentrifuge tubes and 250  $\mu$ l of filter-sterilized papain, pepsin, or trypsin (final concentration of 10 mg/ml) (Sigma Chemical Co., St. Louis, MO) were individually added to cells, cell-free culture supernatants, and freeze-dried concentrated supernatant (resuspended in 5 mM phosphate buffer, pH 6.5) of *C. piscicola* 2762. These were incubated for 1 h at 37°C and then 75  $\mu$ l portions were tested for activity against *L. monocytogenes* as described above. Untreated cells, cell-free supernatant, and freeze-dried supernatant served as controls.

The relative acidogenic potentials of *C. piscicola* and *L. monocytogenes* were examined by comparing the pH changes accompanying monoculture growth of the five isolates in BHI (pH 7.3). Overnight cultures of individual isolates were transferred in 0.1-ml portions to sets of test tubes containing 10 ml of fresh medium. The tubes were incubated at 4, 12, or 19°C. Periodically, one tube per isolate was removed and the pH determined using a pH meter calibrated against a pH 7.00 standard.

A series of studies were performed to assess the effects of nutritional status on the ability of *C. piscicola* 2762 to suppress *L. monocytogenes* in broth cultures. The role of growth factors was assessed by comparing the suppression of *L. monocytogenes* in BHI co-cultures (12°C, pH 7.0, 0.5% NaCl) containing 0.5 and 2.0% yeast extract. The cultures were inoculated, incubated, and plated on BHIA and VJA as described above. The effect of nutrient density was assessed in a similar manner by comparing cultures (12°C, pH 7.0, 0.5% NaCl) containing 1 $\times$ , 3 $\times$ , 6 $\times$ , and 9 $\times$  BHI. In these experiments, *C. piscicola*-only controls were also examined to determine its growth potential at elevated nutrient concentrations.

The effect of oxygen availability on the ability of *C. piscicola* 2762 to suppress *L. monocytogenes* was explored by inoculating cultures (4°C, pH 7.0, 0.5% NaCl) as previously described, and incubating them at varying shaker speeds (0, 250, or 350 rpm). The data were compared with those obtained for cultures incubated at 150 rpm.

The potential for catalase to decrease the antilisterial activity of *C. piscicola* 2762 was investigated by adding filter-sterilized catalase (Sigma) to 250-ml Erlenmeyer flasks containing 50 ml of sterile BHI to achieve a concentration of 0.5 mg/ml. BHI without the enzyme served as the control. The flasks were inoculated, incubated at 19°C, and cultured as previously described. The retention of high levels of enzyme activity at the end of the incubation period was verified by determining the ability of the medium to react with a hydrogen peroxide solution.

## RESULTS

#### Co-culture studies

While small differences in LPD and GT values were observed when *L. monocytogenes* was cultured alone or in

the presence of *C. piscicola*, the primary effect of *C. piscicola* on *L. monocytogenes* was a suppression of the pathogen's MPD (Fig. 1). The inhibitory effect was roughly equivalent for the two *C. piscicola* strains. The extent of the MPD suppression was dependent on the interaction of the three culture conditions (Tables 1 and 2). This can be readily observed by comparing the differentials between the MPDs for the *L. monocytogenes* controls and their corresponding co-cultures ( $MPD_{\text{control}} - MPD_{\text{co-culture}}$ ) (Fig. 2). The ability of *C. piscicola* to suppress the growth of *L. monocytogenes* was favored by low temperature, low NaCl content, and pH values in the neutral to slightly acidic range.

*Carnobacterium piscicola* 2762 was originally selected for inclusion in this study as a bacteriocin-negative strain on the basis of an initial screening of isolates using a spot-on-the-lawn assay. However, the above results prompted a detailed reexamination of the strain. It appears that the isolate produces a bacteriocin that remains associated with the cell and is only released when cells are exposed to a relatively strong acidic environment. The antilisterial activity was eliminated by treatment with trypsin and reduced by pepsin or papain.

The ability of both *C. piscicola* strains to suppress *L. monocytogenes* appears to be related to the relative growth rates of the two species under any specific set of cultural conditions. This relationship was examined quantitatively comparing the *L. monocytogenes* MPD differentials between the control and co-cultures ( $MPD_{\text{control}} - MPD_{\text{co-culture}}$ ) against the co-cultures'  $T_{10}$  ratios (Fig. 3). The  $T_{10}$  ratio was used as a means of quantitatively expressing the relative initiation and rate of growth of *L. monocytogenes* and *C. piscicola* in the co-culture. It was defined as the time for a 10-fold increase based on the VJA counts divided by the time to a 10-fold increase based on the BHIA counts. If *L. monocytogenes* grew as fast or faster than *C. piscicola*, the  $T_{10}$  ratio would be approximately 1. Conversely, if *C. piscicola* grew more rapidly, the  $T_{10}$  ratio would be greater than 1. A 10-fold increase encompasses the lag phase and the start of the exponential growth phase. This value was selected because,

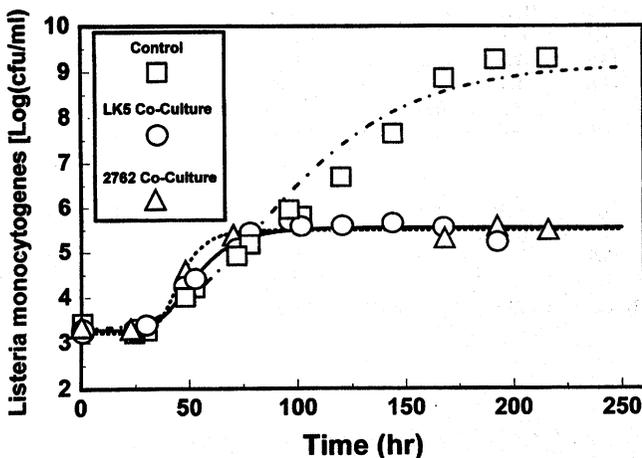


FIGURE 1. Example of the effects of *Carnobacterium piscicola* LK5 and 2762 on the growth of *Listeria monocytogenes* Scott A; cultural conditions, 12°C, pH 6, 25 g of NaCl/liter.

under some of the cultural conditions, the increase in *L. monocytogenes* populations during exponential growth was less than 100-fold. This analysis indicated that the faster *L. monocytogenes* grew in relation to *C. piscicola*, the smaller was the extent of the MPD suppression.

Since *C. piscicola* 2762 was ultimately identified as producing a cell-associated bacteriocin, two additional bacteriocin-negative strains were identified and examined for their ability to suppress the MPD of *L. monocytogenes*. A single set of cultural conditions (4°C, pH 7, 0.5% NaCl) that produced a substantial suppression of MPD with *C. piscicola* 2762 and LK5 was examined (Fig. 4). A substantial suppression of *L. monocytogenes* growth was noted with both of the bacteriocin-negative strains. While there appeared to be a small enhancement of suppression with the bacteriocin-producing strains, overall the results indicated that little of the ability of these *Carnobacterium* isolates to suppress the growth of *L. monocytogenes* appears to be attributable to bacteriocin production.

#### Further characterization of the antilisterial activity of *C. piscicola* 2762

A series of supplemental studies was performed with *C. piscicola* 2762 to determine the mechanism underlying its ability to suppress the growth of *L. monocytogenes*. Acid production by *C. piscicola* and *L. monocytogenes* was examined by monitoring the pH changes accompanying growth in BHI with an initial pH of 7.3 (Fig. 5). The four *C. piscicola* strains were not strong acid producers; the pH only decreased to 5.3 to 5.6. This is similar to the pH depression accompanying the growth of *L. monocytogenes*. Differences in the time that the lowest pH was reached were observed among the *C. piscicola* isolates and between the species. In the latter case, the greatest differences occurred at 4°C, reflecting the effect of temperature on the growth rates of the two microorganisms.

The possibility that 2762 was producing peroxide was examined by incorporating catalase into co-cultures (19°C, pH 7, 0.5% NaCl). This produced no diminution of the MPD suppression (data not shown), despite the medium having demonstrable enzyme activity at the end of the incubation period.

The possibility that the growth of *C. piscicola* was depleting available oxygen for the slower growing *L. monocytogenes* was examined by incubating the co-cultures and controls (4°C, pH 7, 0.5% NaCl) incubated at different shaker speeds (0, 150, 250, 350 rpm). It could be anticipated that decreasing the speed of the shaker would exacerbate any oxygen deficiency, while increasing the rate of agitation would increase oxygen availability (12). However, the organisms grew equivalently at the different shaker speeds and there was no difference in the extent of MPD suppression (data not shown). At least within the ability of altering shaker speed to increase or diminish effects related to oxygen availability, it does not appear that the suppression of *L. monocytogenes* by *C. piscicola* is related to oxygen content.

Two sets of nutrient supplementation studies (cultural conditions: 12°C, pH 7, 0.5% NaCl) were performed to determine if the suppression of *L. monocytogenes* was related to nutrient depression. In the first set, BHI was

TABLE 1. Effect of temperature, pH, and sodium chloride on the ability of *Carnobacterium piscicola* LK5 to influence the growth kinetics of *Listeria monocytogenes* Scott A

Temp. (°C)	pH	NaCl (g/liter)	Growth parameters, <sup>a</sup> mean (SD)															
			<i>L. monocytogenes</i> on VGA <sup>b</sup>				<i>L. monocytogenes</i> / <i>C. piscicola</i> LK5 co-cultures on VGA				<i>L. monocytogenes</i> / <i>C. piscicola</i> LK5 co-cultures on BHIA <sup>b</sup>							
			n	EGR	GT	LPD	MPD	n	EGR	GT	LPD	MPD	n	EGR	GT	LPD	MPD	
19	5	5	2	0.043 (0.001)	7.0 (0.2)	59.0 (9.3)	9.3 (0.4)	2	0.052 (0.002)	5.8 (0.3)	51.6 (0.5)	9.1 (0.2)	2	0.055 (0.002)	5.5 (0.2)	44.9 (0.02)	8.7 (0.1)	
			25	2	0.085 (0.007)	3.6 (0.3)	74.9 (2.2)	8.9 (0.0)	1	0.069 — <sup>c</sup>	4.4 —	83.3 —	8.9 —	1	0.071 —	4.3 —	86.3 —	8.8 —
		45	2	0.054 (0.007)	5.7 (0.7)	122.7 (0.1)	7.9 (0.0)	2	0.061 (0.004)	5.0 (0.3)	111.6 (0.7)	8.1 (0.0)	2	0.059 (0.002)	5.1 (0.1)	113.6 (4.7)	8.1 (0.2)	
	6	5	2	0.215 (0.001)	1.4 (0.0)	11.1 (0.1)	9.6 (0.1)	2	0.197 (0.007)	1.5 (0.1)	6.6 (1.1)	7.2 (0.0)	2	0.219 (0.002)	1.4 (0.0)	6.7 (0.5)	9.2 (0.0)	
			25	2	0.211 (0.002)	1.4 (0.0)	11.6 (0.1)	9.6 (0.1)	2	0.208 (0.018)	1.5 (0.1)	10.8 (1.5)	7.7 (0.2)	2	0.190 (0.002)	1.6 (0.0)	10.2 (0.6)	9.0 (0.2)
		45	2	0.138 (0.007)	2.2 (0.1)	13.0 (0.2)	9.5 (0.2)	2	0.162 (0.001)	1.9 (0.0)	13.1 (0.1)	9.3 (0.0)	2	0.161 (0.007)	1.9 (0.1)	14.7 (0.6)	9.3 (0.0)	
	7	5	2	0.224 (0.007)	1.3 (0.0)	8.2 (0.3)	9.7 (0.0)	2	0.210 (0.004)	1.4 (0.0)	6.6 (0.6)	7.2 (0.0)	2	0.246 (0.007)	1.2 (0.0)	5.5 (0.3)	9.6 (0.2)	
			25	2	0.220 (0.006)	1.4 (0.0)	9.5 (0.0)	9.8 (0.0)	2	0.215 (0.002)	1.4 (0.0)	7.8 (1.3)	8.2 (0.3)	2	0.214 (0.000)	1.4 (0.0)	7.0 (1.3)	9.1 (0.2)
		45	2	0.210 (0.006)	1.4 (0.0)	11.2 (0.7)	9.7 (0.0)	2	0.205 (0.016)	1.5 (0.1)	12.5 (0.6)	9.1 (0.1)	2	0.197 (0.001)	1.5 (0.0)	12.6 (1.4)	9.2 (0.0)	
	12	5	5	2	0.000 (0.000)	—	—	—	2	0.000 (0.000)	—	—	—	2	0.037 (0.001)	8.2 (0.2)	73.7 (0.1)	8.7 (0.0)
				25	2	0.016 (0.004)	19.9 (5.2)	533.4 (3.0)	6.5 (0.0)	2	0.022 (0.000)	13.9 (0.3)	356.2 (3.4)	8.3 (0.0)	2	0.014 (0.000)	21.1 (0.4)	278.8 (7.5)
			45	2	0.000 (0.000)	—	—	—	2	0.000 (0.000)	—	—	—	2	0.000 (0.000)	—	—	—
6		5	2	0.047 (0.002)	6.4 (0.3)	46.9 (17.4)	9.3 (0.3)	2	0.063 (0.04)	4.8 (0.3)	28.5 (1.5)	5.4 (0.0)	2	0.077 (0.001)	3.9 (0.0)	15.7 (2.6)	9.2 (0.0)	
			25	2	0.048 (0.000)	6.3 (0.0)	43.3 (1.7)	9.3 (0.1)	2	0.063 (0.001)	4.8 (0.1)	34.0 (0.9)	5.6 (0.1)	2	0.054 (0.000)	5.6 (0.0)	24.0 (2.3)	9.3 (0.2)
		45	2	0.034 (0.002)	8.9 (0.5)	69.3 (1.5)	9.7 (0.0)	2	0.030 (0.001)	9.9 (0.2)	63.9 (0.7)	7.4 (0.1)	2	0.028 (0.000)	10.9 (0.0)	61.6 (0.2)	8.4 (0.0)	
7		5	2	0.064 (0.000)	4.7 (0.0)	27.2 (1.9)	9.7 (0.0)	2	0.071 (0.001)	4.2 (0.1)	22.6 (1.1)	5.5 (0.0)	2	0.089 (0.005)	3.4 (0.2)	12.0 (1.6)	9.4 (0.0)	
			25	2	0.060 (0.001)	5.0 (0.0)	28.5 (1.8)	9.7 (0.1)	2	0.057 (0.001)	5.1 (0.1)	21.9 (1.5)	5.9 (0.0)	2	0.073 (0.002)	4.1 (0.1)	14.5 (0.6)	9.4 (0.2)
		45	2	0.048 (0.001)	6.2 (0.1)	35.8 (1.1)	10.0 (0.1)	2	0.026 (0.004)	11.6 (1.5)	12.9 (4.7)	7.3 (0.4)	2	0.061 (0.001)	4.9 (0.1)	28.1 (0.2)	9.0 (0.0)	
4		5	5	2	0.000 (0.000)	—	—	—	2	0.000 (0.000)	—	—	—	2	0.028 (0.002)	11.0 (1.0)	147.6 (6.6)	8.4 (0.1)
				25	2	0.000 (0.000)	—	—	—	2	0.000 (0.000)	—	—	—	2	0.000 (0.000)	—	—
			45	2	0.000 (0.000)	—	—	—	2	0.000 (0.000)	—	—	—	2	0.000 (0.000)	—	—	—
	6	5	2	0.028 (0.002)	10.8 (0.9)	89.6 (7.1)	9.4 (0.1)	2	0.017 (0.007)	21.7 (9.3)	62.3 (13.3)	3.8 (0.1)	2	0.043 (0.006)	7.1 (1.0)	43.3 (0.7)	9.1 (0.1)	
			25	2	0.021 (0.000)	14.2 (0.1)	86.6 (6.7)	8.9 (0.1)	2	0.012 (0.002)	24.2 (4.5)	31.9 (9.2)	4.6 (0.2)	2	0.029 (0.002)	10.3 (0.8)	36.1 (3.9)	8.7 (0.0)
		45	2	0.028 (0.002)	10.7 (0.6)	89.4 (10.8)	8.8 (0.1)	2	0.032 (0.014)	11.8 (5.2)	70.1 (13.0)	4.8 (0.3)	2	0.035 (0.005)	8.7 (1.3)	63.4 (10.6)	8.4 (0.1)	
	7	5	2	0.032 (0.000)	9.5 (0.1)	78.0 (2.6)	10.6 (0.0)	2	0.614 (0.589)	5.6 (5.4)	73.6 (46.3)	4.3 (0.0)	2	0.073 (0.004)	4.2 (0.2)	56.2 (4.8)	9.5 (0.1)	
			25	2	0.020 (0.000)	15.3 (0.2)	62.2 (2.0)	9.0 (0.0)	2	0.038 (0.006)	8.2 (1.2)	82.5 (0.9)	4.4 (0.0)	2	0.044 (0.001)	6.8 (0.2)	57.9 (2.1)	10.0 (0.5)
		45	2	0.026 (0.001)	11.7 (0.3)	106.5 (5.0)	9.5 (0.6)	2	0.024 (0.002)	12.9 (1.0)	95.6 (1.9)	6.9 (0.4)	2	0.027 (0.003)	11.4 (1.1)	96.3 (1.1)	9.4 (0.2)	

<sup>a</sup> EGR, exponential growth rate; GT, generation time; LPD, lag-phase duration; MPD, maximum population density.

<sup>b</sup> VGA, Vogel Johnson agar; BHIA, brain heart infusion agar.

<sup>c</sup> No growth.

supplemented with additional yeast extract (final concentration, 20 g/liter) to increase micronutrient availability. No change in MPD suppression was observed (data not shown). In the second study, *L. monocytogenes* and *C. piscicola*, alone and in combination, were cultured in media having

1×, 3×, 6×, and 9× levels of BHI. Neither *L. monocytogenes* nor *C. piscicola* grew in the 9× BHI, presumably due to water activity depression resulting from the high solute content. At the lower BHI levels, both *L. monocytogenes* and *C. piscicola* controls grew, reaching MPD levels of approxi-

TABLE 2. Effect of temperature, pH, and sodium chloride on the ability of *Carnobacterium piscicola* 2762 to influence the growth kinetics of *Listeria monocytogenes* Scott A

Temp. (°C)	pH	NaCl (g/liter)	Growth parameters, <sup>a</sup> mean (SD)																
			<i>L. monocytogenes</i> on VGA <sup>b</sup>				<i>L. monocytogenes</i> /C. <i>piscicola</i> 2762 co-cultures on VGA					<i>L. monocytogenes</i> /C. <i>piscicola</i> 2762 co-cultures on BHIA <sup>b</sup>							
			n	EGR	GT	LPD	MPD	n	EGR	GT	LPD	MPD	n	EGR	GT	LPD	MPD		
19	5	5	2	0.043 (0.000)	7.0 (0.0)	112.2 (4.3)	8.6 (0.0)	2	0.362 (0.318)	3.4 (3.36)	114.2 (10.9)	4.9 (0.0)	2	0.095 (0.012)	3.2 (0.4)	82.4 (4.1)	7.6 (0.0)		
			25	2	0.037 (0.001)	8.3 (0.1)	162.7 (7.8)	7.7 (0.0)	2	0.039 (0.003)	7.9 (0.6)	177.7 (31.4)	7.6 (0.3)	2	0.032 (0.003)	9.5 (0.8)	178.2 (32.8)	7.6 (0.4)	
		45	2	0.015 (0.000)	20.4 (0.1)	214.2 (3.4)	7.2 (0.0)	2	0.011 (0.003)	28.5 (6.9)	348.0 (105.2)	6.7 (0.2)	2	0.009 (0.003)	37.2 (11.5)	359.2 (114.4)	7.0 (0.1)		
	6	5	4	4	0.163 (0.020)	1.9 (0.2)	14.7 (0.9)	9.3 (0.2)	4	0.160 (0.031)	1.9 (0.3)	13.6 (1.1)	6.7 (0.1)	4	0.202 (0.008)	1.5 (0.1)	10.2 (2.1)	9.1 (0.1)	
				25	2	0.119 (0.000)	2.5 (0.0)	13.9 (0.2)	9.5 (0.0)	2	0.151 (0.000)	2.0 (0.0)	16.8 (0.2)	8.3 (0.0)	2	0.132 (0.001)	2.3 (0.0)	15.4 (0.2)	8.8 (0.0)
		45	2	0.114 (0.010)	2.7 (0.2)	25.6 (4.0)	9.7 (0.2)	2	0.124 (0.000)	2.8 (0.0)	31.6 (0.1)	8.7 (0.0)	2	0.096 (0.002)	3.2 (0.1)	33.4 (1.0)	9.9 (0.0)		
	7	5	2	2	0.236 (0.003)	1.3 (0.0)	13.4 (0.1)	9.6 (0.1)	2	0.170 (0.001)	1.8 (0.1)	6.8 (0.2)	7.6 (0.1)	2	0.210 (0.000)	1.4 (0.0)	5.4 (0.1)	9.7 (0.0)	
				25	2	0.226 (0.003)	1.3 (0.0)	14.6 (0.1)	9.3 (0.0)	2	0.176 (0.013)	1.7 (0.1)	11.0 (0.9)	8.3 (0.1)	2	0.187 (0.002)	1.6 (0.0)	8.8 (0.1)	9.9 (0.0)
		45	3	0.130 (0.005)	2.3 (0.1)	13.7 (0.2)	9.1 (0.2)	4	0.144 (0.004)	2.1 (0.1)	13.4 (1.3)	8.9 (0.1)	4	0.145 (0.013)	2.1 (0.2)	15.3 (0.7)	9.2 (0.0)		
	12	5	5	2	0.000 (0.000)	— <sup>c</sup> —	— —	— —	2	0.000 (0.000)	— —	— —	— —	2	0.059 (0.001)	5.1 (0.0)	228.3 (1.1)	7.9 (0.1)	
				25	2	0.000 (0.000)	— —	— —	— —	2	0.000 (0.000)	— —	— —	— —	2	0.000 (0.000)	— —	— —	— —
				45	2	0.000 (0.000)	— —	— —	— —	2	0.000 (0.000)	— —	— —	— —	2	0.000 (0.000)	— —	— —	— —
6		5	2	2	0.081 (0.001)	3.7 (0.1)	34.2 (1.8)	9.2 (0.1)	2	0.074 (0.005)	4.1 (0.3)	27.1 (2.5)	5.5 (0.1)	2	0.110 (0.001)	2.7 (0.0)	12.4 (0.1)	9.0 (0.0)	
				25	1	0.067 —	4.5 —	31.5 —	8.9 —	2	0.108 (0.048)	3.5 (1.5)	32.7 (7.2)	5.5 (0.0)	2	0.103 (0.05)	2.9 (0.1)	17.8 (0.9)	8.6 (0.0)
		45	2	0.035 (0.000)	8.6 (0.1)	34.3 (0.3)	9.0 (0.0)	2	0.060 (0.004)	5.0 (0.3)	39.8 (0.9)	6.1 (0.0)	2	0.065 (0.001)	4.6 (0.1)	27.4 (1.2)	8.4 (0.1)		
7		5	2	2	0.082 (0.004)	3.7 (0.2)	21.8 (4.8)	9.0 (0.1)	2	0.060 (0.000)	5.0 (0.0)	16.3 (3.0)	6.5 (0.2)	2	0.118 (0.000)	2.6 (0.0)	11.0 (0.6)	9.3 (0.1)	
				25	2	0.079 (0.003)	3.8 (0.2)	23.1 (2.7)	9.6 (0.1)	3	0.080 (0.005)	3.8 (0.2)	20.3 (2.0)	7.0 (0.1)	2	0.063 (0.005)	4.8 (0.4)	6.4 (1.0)	9.5 (0.1)
		45	2	0.089 (0.001)	3.4 (0.0)	35.7 (0.1)	9.3 (0.0)	2	0.063 (0.004)	4.8 (0.3)	29.4 (4.1)	9.0 (0.1)	2	0.087 (0.003)	3.5 (0.1)	40.9 (0.4)	9.1 (0.0)		
4		5	5	2	0.000 (0.000)	— —	— —	— —	2	0.000 (0.000)	— —	— —	— —	2	0.025 (0.006)	12.8 (3.0)	207.6 (41.4)	9.0 (0.1)	
				25	2	0.000 (0.000)	— —	— —	— —	2	0.000 (0.000)	— —	— —	— —	2	0.000 (0.000)	— —	— —	— —
				45	2	0.000 (0.000)	— —	— —	— —	2	0.000 (0.000)	— —	— —	— —	2	0.000 (0.000)	— —	— —	— —
	6	5	2	2	0.023 (0.000)	12.9 (0.1)	132.0 (2.8)	9.3 (0.1)	2	0.004 (0.000)	76.2 (0.7)	32.7 (13.1)	4.7 (0.1)	2	0.054 (0.002)	5.6 (0.2)	42.4 (2.7)	9.0 (0.1)	
				25	2	0.025 (0.001)	12.3 (0.1)	89.0 (7.6)	9.2 (0.3)	1	0.019 —	15.7 —	104.7 —	4.6 —	2	0.038 (0.004)	8.1 (0.9)	57.8 (5.4)	9.3 (0.2)
		45	2	0.017 (0.001)	18.0 (1.3)	262.8 (20.1)	8.4 (0.3)	2	0.053 (0.000)	14.1 (0.3)	167.9 (4.0)	5.2 (0.0)	2	0.020 (0.000)	14.9 (0.0)	185.8 (3.8)	8.8 (0.0)		
	7	5	6	6	0.027 (0.002)	11.3 (1.0)	88.0 (9.9)	9.0 (0.5)	5	0.050 (0.041)	9.9 (5.4)	73.1 (15.9)	4.5 (0.1)	6	0.084 (0.031)	4.1 (1.5)	56.5 (22.4)	9.3 (0.1)	
				25	1	0.035 (0.002)	8.8 (0.4)	87.7 (10.7)	9.3 (0.1)	1	0.030 (0.008)	10.9 (2.7)	79.4 (0.0)	5.6 (0.1)	2	0.063 (0.005)	4.8 (0.3)	83.7 (5.6)	9.1 (0.0)
		45	2	0.020 (0.000)	15.0 (0.3)	111.9 (6.2)	9.2 (0.0)	2	0.017 (0.002)	18.0 (2.1)	89.6 (9.0)	7.1 (0.3)	2	0.028 (0.001)	11.1 (0.1)	124.6 (7.3)	9.0 (0.0)		

<sup>a</sup> EGR, exponential growth rate; GT, generation time; LPD, lag-phase duration; MPD, maximum population density.

<sup>b</sup> VGA, Vogel Johnson agar; BHIA, brain heart infusion agar.

<sup>c</sup> No growth.

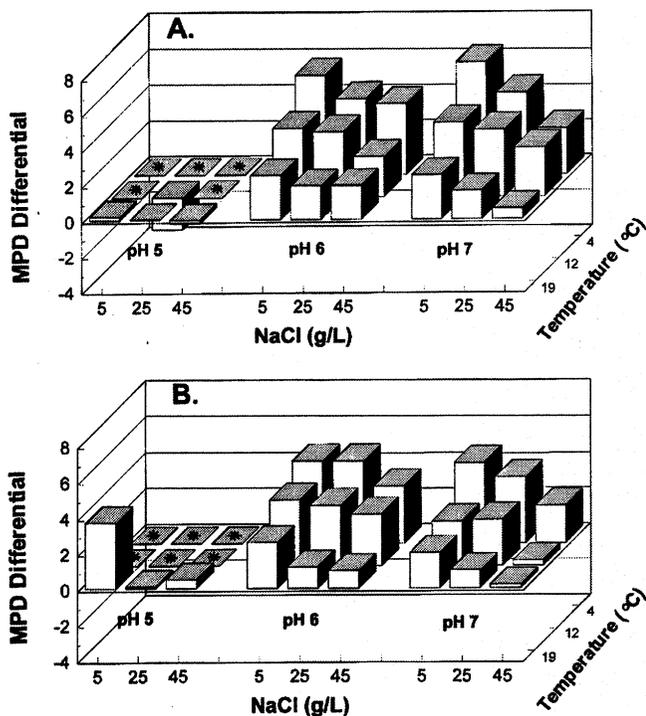


FIGURE 2. Effect of *Carnobacterium piscicola* LK5 (A) and 2762 (B) on the maximum population density (MPD) reached by *Listeria monocytogenes* under various conditions of temperature, initial pH, and sodium chloride content. MPD differential, (MPD control) – (MPD co-culture); \*, no growth of *L. monocytogenes* in control or co-cultures. As the difference of two log values (and the ratio of two untransformed values), the MPD differential term is unitless.

mately  $10^9$  CFU/ml (Fig. 6a). In all cases, *C. piscicola* grew faster than *L. monocytogenes*. When grown in co-culture, the extent of *L. monocytogenes* MPD suppression was decreased as the BHI concentration was increased (Fig. 6b). While the additional BHI did not totally reverse the MPD suppression, the data suggest that the suppression of *L.*

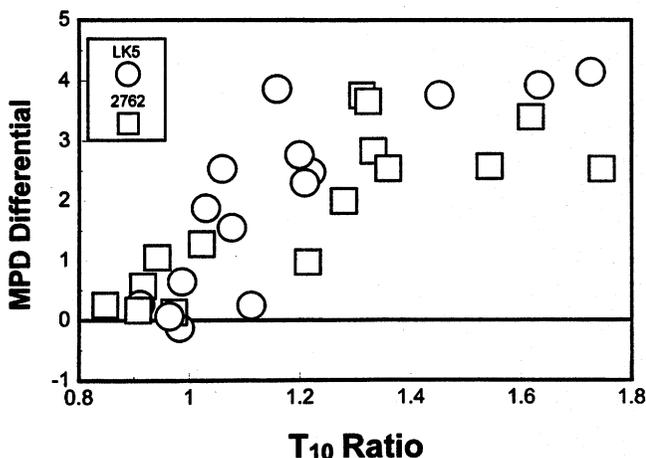


FIGURE 3. Relationship between the extent of maximum population density (MPD) suppression of *Listeria monocytogenes* Scott A and the relative growth rates of the *L. monocytogenes* and *C. piscicola* LK5 and 2762. MPD differential, (MPD control) – (MPD co-culture);  $T_{10}$  Ratio =  $T_{10}$  based on VJA counts/ $T_{10}$  based on BHI counts;  $T_{10}$ , time to a 10-fold increase in population density.

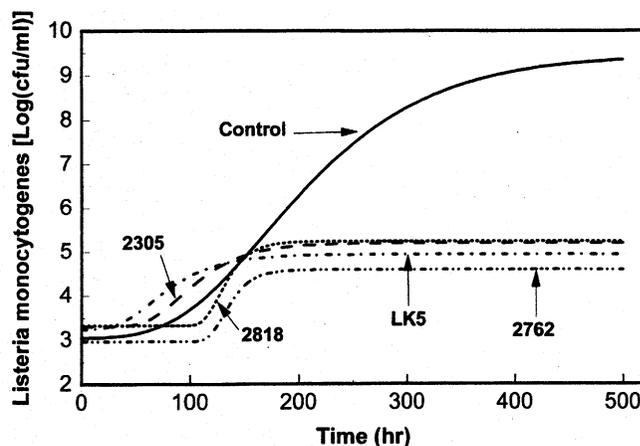


FIGURE 4. Growth of *Listeria monocytogenes* Scott A in the presence of *Carnobacterium piscicola* strains that excrete a bacteriocin (LK5), produce a cell-associated bacteriocin (2762), and do not produce a bacteriocin (2305, 2818). Growth curves based on mean of Gompertz parameters from at least two independent cultures. Culture conditions,  $4^{\circ}\text{C}$ , pH 7, 5 g of NaCl/liter.

*monocytogenes* may be related, at least in part, to nutrient depletion. Determination of which nutrient this is will require additional studies.

## DISCUSSION

When microorganisms share an environment, they can interact in three ways. First, the two microorganisms can occupy separate niches and have totally separate requirements. In such an instance it would be expected that each would grow virtually independent of the other. Second, one or both of the microorganisms can derive a benefit from the other, which would be reflected an increase in either the rate or extent of growth. This is often the case when a microorganism produces extracellular enzymes or growth factors needed by the other. Finally, microorganisms may compete for the same niche, or one microorganism may produce an antagonistic extracellular agent or modify the environment such that the other is inhibited. The interaction between *L. monocytogenes* and *C. piscicola* clearly belongs to the third category, competition.

The effect of *C. piscicola* on *L. monocytogenes* was largely limited to a suppression of the MPD, with the extent of the effect being dependent on the three culture parameters (Tables 1 and 2). The extent of the suppression appears to be a function of the relative growth rates of the two bacteria (Fig. 3), with cultural conditions that favor the growth of *L. monocytogenes* decreasing the suppression by *C. piscicola*. The timing of *L. monocytogenes* growth suppression generally coincided with *C. piscicola* entering early stationary-phase growth. This suggests either depletion of a critical nutrient or the production of an inhibitory extracellular agent (e.g., an acid, peroxide, or bacteriocin) associated with the microorganism reaching a specific population density. The pH decreases produced by the four *C. piscicola* strains were similar and occurred at a time in the growth cycle roughly corresponding to the initiation of MPD suppression. How-

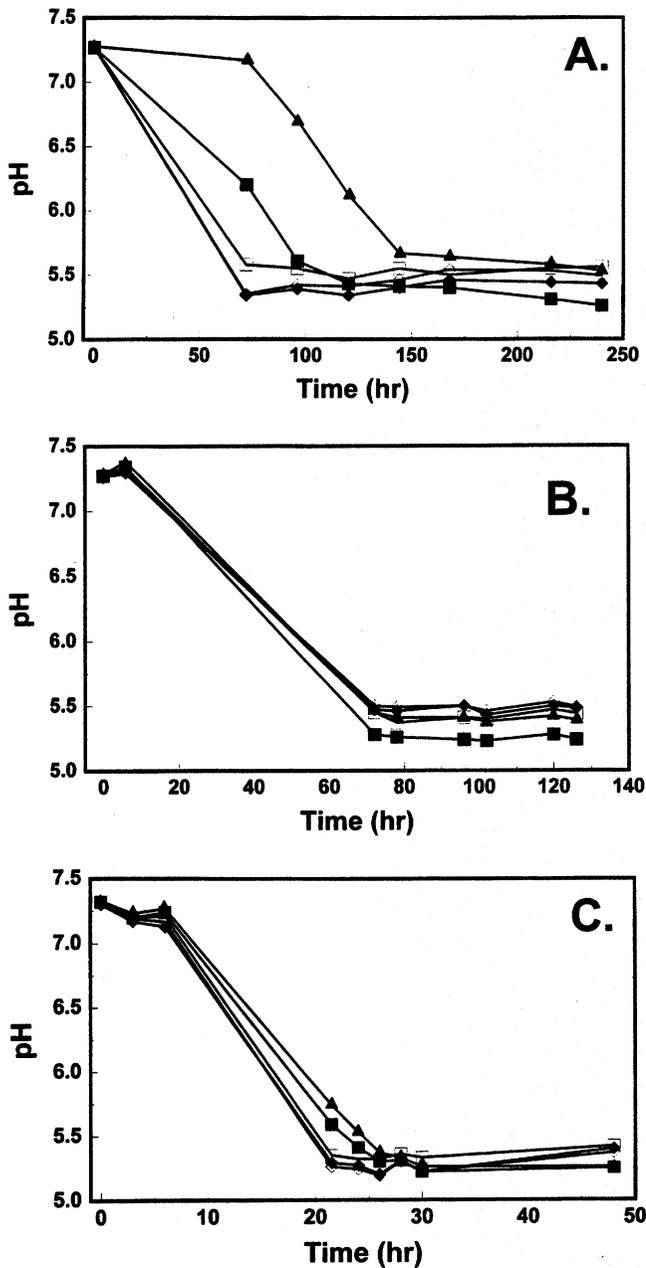


FIGURE 5. The decline in pH accompanying the growth of *Carnobacterium piscicola* and *Listeria monocytogenes* monocultures (10 ml of brain heart infusion broth) incubated at 4° (A), 12°C (B), and (C) 19°C. *C. piscicola* 2818  $\blacklozenge$ , *C. piscicola* 2762  $\blacklozenge$ , *C. piscicola* 2305  $\blacksquare$ , *C. piscicola* LK5  $\square$ , *L. monocytogenes* Scott A  $\blacktriangle$ .

ever, it is unlikely that the magnitude of the pH decline is great enough to halt the exponential growth of *L. monocytogenes* (4). Furthermore, Buchanan and Klawitter (2), using co-cultures of *C. piscicola* LK5 and *L. monocytogenes* Scott A grown in tryptic soy broth with and without glucose, demonstrated that the suppression of *L. monocytogenes* MPD was not a function of acid production by *C. piscicola*. Likewise, there was little evidence to indicate that the effect was primarily due to the accumulation of another inhibitory extracellular agent. However, at least part of the MPD suppression appeared to be reduced when the two microorganisms were cultured in media with increased nutrient

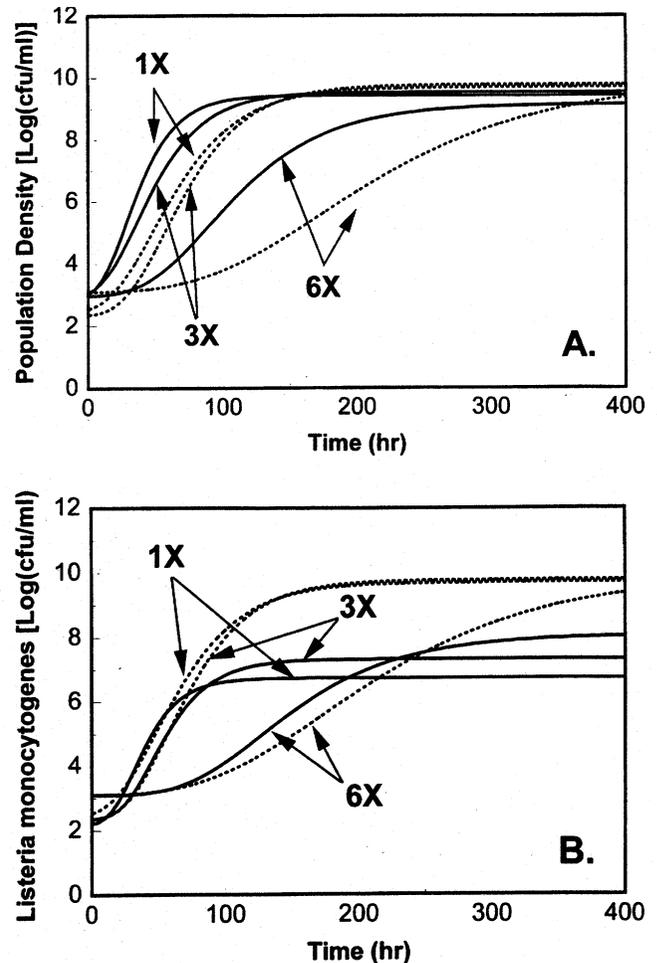


FIGURE 6. Growth of *Carnobacterium piscicola* 2762 and *Listeria monocytogenes* Scott A in 1X, 3X, and 6X brain heart infusion broth. Culture conditions, 12°C, pH 7, 5 g of NaCl/liter (A) Growth of *C. piscicola* (—) and *L. monocytogenes* (---) as monocultures. (B) Growth of *L. monocytogenes* as a monoculture (---) and as a co-culture with *C. piscicola* (—).

levels, suggesting a mechanism involving nutrient depletion. Interestingly, our early studies with *C. piscicola* LK5 (2) found that the initial ratio of *C. piscicola* to *L. monocytogenes* had relatively little effect on the level of MPD suppression, even though this would influence the time in the growth cycle of *L. monocytogenes* that coincided with *C. piscicola* reaching stationary growth.

The suppression of MPD by *C. piscicola* observed in the current study is similar to that observed previously for *C. piscicola* LK5 in both media and foods (2, 3). Only in irradiation-sterilized raw ground beef was a bacteriocidal effect observed (3). At least in the media system employed, the current results suggest that little of the effect of the tested *C. piscicola* isolates could be attributed to bacteriocin production (Fig. 4). While it is not possible to absolutely prove that strains 2305 and 2818 do not produce bacteriocins, both were consistently negative when examined exhaustively for antimicrobial production. There is a possibility that bacteriocin production plays a greater role in a solid matrix where the antimicrobial agent would be likely to achieve greater local concentrations. Mathieu et al. (7)

evaluated the ability of bacteriocin-producing *C. piscicola* CP5, a bacteriocin-negative variant of CP5, and bacteriocin-nonproducing *C. piscicola* CP7 to affect the growth of *L. monocytogenes* in microbiological medium and skim milk. They observed that CP5 was either bactericidal or bacteriostatic, depending on the substrate and the incubation temperature. However, both the bacteriocin-negative CP5 variant and the nonproducing CP7 were able to produce a marked suppression of *L. monocytogenes* MPD.

It appears that in addition to any effects that bacteriocins produced by different *Carnobacterium* isolates may have, the genus has a general characteristic of suppressing *L. monocytogenes* growth under environmental conditions that are associated with many refrigerated foods (2). The current results suggest that this may be due to nutrient depletion. It is unlikely that the effect is due to a generalized nutrient deficiency. Instead, the more rapidly growing *C. piscicola* may produce an insufficiency of a limiting nutrient for *L. monocytogenes*. The identification of the specific limiting component will require additional research. Additional studies are currently underway with *Pseudomonas fluorescens* to determine if *L. monocytogenes* suppression can be produced by other psychrotrophs commonly associated with refrigerated food products of animal origin.

#### REFERENCES

1. Ahn, C., and M. E. Stiles. 1990. Plasmid associated bacteriocin production by a strain of *Carnobacterium piscicola* from meat. *Appl. Environ. Microbiol.* 56:2503-2510.
2. Buchanan, R. L., and L. A. Klawitter. 1992. Characterization of a lactic acid bacterium, *Carnobacterium piscicola* LK5, with activity against *Listeria monocytogenes* at refrigeration temperatures. *J. Food Safety* 12:199-217.
3. Buchanan, R. L., and L. A. Klawitter. 1992. Effectiveness of *Carnobacterium piscicola* LK5 for controlling the growth of *Listeria monocytogenes* Scott A in refrigerated foods. *J. Food Safety* 12:219-236.
4. Buchanan, R. L., H. G. Stahl, and R. C. Whiting. 1989. Effects and interactions of temperature, pH, atmosphere, sodium chloride, and sodium nitrite on the growth of *Listeria monocytogenes*. *J. Food Prot.* 52:844-851.
5. Gibson, A. M., N. Bratchell, and T. A. Roberts. 1987. The effect of sodium chloride and temperature on the rate and extent of growth of *Clostridium botulinum* type A in pasteurized pork slurry. *J. Appl. Bacteriol.* 62:479-490.
6. Lewus, C. B., A. Kaiser, and T. J. Montville. 1991. Inhibition of food-borne bacterial pathogens by bacteriocins from lactic acid bacteria isolated from meat. *Appl. Environ. Microbiol.* 57:1683-1688.
7. Mathieu, F., M. Michel, A. Lebrhi, and G. Lefebvre. 1994. Effect of the bacteriocin carnocin CP5 and of the producing strain *Carnobacterium piscicola* CP5 on the viability of *Listeria monocytogenes* ATCC 15313 in salt solution, broth, and skimmed milk, at various incubation temperatures. *Int. J. Food Microbiol.* 22:155-172.
8. Mathieu, F., M. Michel, and G. Lefebvre. 1993. Properties of a bacteriocin produced by *Carnobacterium piscicola* CP5. *Biotech. Lett.* 15:587-590.
9. Milliere, J. B., M. Michel, F. Mathieu, and G. Lefebvre. 1994. Presence of *Carnobacterium* spp. in French surface mould-ripened soft-cheese. *J. Appl. Bacteriol.* 76:264-269.
10. Pilet, M.-F., X. Dousset, R. Barre, G. Novel, M. Desmazeaud, and J.-C. Piard. 1995. Evidence for two bacteriocins produced by *Carnobacterium piscicola* and *Carnobacterium divergens* isolated from fish and active against *Listeria monocytogenes*. *J. Food Prot.* 58:256-262.
11. Schillinger, U., and W. H. Holzapfel. 1990. Antibacterial activity of carnobacteria. *Food Microbiol.* 7:305-310.
12. Sikyta, B. 1983. *Methods in industrial microbiology*, p. 109-144. Ellis Howard Limited, Chichester, England.
13. Stoffels, G., H. Sahl, and A. Gudmundsdottir. 1993. Carnocin U149, a potential biopreservative produced by *Carnobacterium piscicola*: large scale purification and activity against various gram-positive bacteria including *Listeria* spp. *Int. J. Food Microbiol.* 20:199-210.
14. Tagg, J. R., A. S. Dajani, and L. W. Wanamaker. 1976. Bacteriocins of gram-positive bacteria. *Bacteriol. Rev.* 40:722-756.