

CHARACTERIZATION OF A LACTIC ACID BACTERIUM,
CARNOBACTERIUM PISCICOLA LK5, WITH ACTIVITY
AGAINST *LISTERIA MONOCYTOGENES* AT REFRIGERATION
TEMPERATURES

ABSTRACT

A lactic acid bacterium (LK5) originally isolated from raw ground beef was characterized in relation to its ability to inhibit the growth of Listeria monocytogenes. The isolate, which was identified as Carnobacterium piscicola, inhibited the growth of 17 of 21 strains of Listeria (L. monocytogenes, L. innocua, L. ivanovii, L. welshimeri, and L. grayii). Its activity was not due to either acid or hydrogen peroxide production, but was related to the production of a heat stable bacteriocin. The isolate was most active against L. monocytogenes at refrigeration temperatures due to the combined effect of the pathogen's increased susceptibility, LK5's rapid growth rate, and enhanced bacteriocin production at low temperatures. Examination of the effect of inoculum ratios in co-cultures of C. piscicola LK5 and L. monocytogenes Scott A indicated that the lactic acid bacterium was active against L. monocytogenes even when the initial level of the pathogen was 100-fold greater. Evaluation of the impact of oxygen availability, initial pH, and sodium chloride content on the effectiveness of LK5 suggested that the isolate could be used to suppress the growth of Listeria in a variety of refrigerated foods.

INTRODUCTION

The realization that foods can play a major role in the transmission of *Listeria monocytogenes* has led to interest in new methods for controlling this psychrotrophic pathogen, particularly in refrigerated foods. Among the techniques being investigated, the use of antagonistic bacteria or bacteriocins produced by these microorganisms has received increased attention during the past several years (Ahn and Stiles 1990; Benkerroum and Sandine 1988; Berry *et al.* 1990; Bhunia *et al.* 1988; Carminati *et al.* 1989; Gouet *et al.* 1978; Harris 1989; Hoover *et al.* 1988, 1989; Lewus *et al.* 1991; Ortel 1989; Pucci *et al.* 1988; Schillinger and Lucke 1989; Schillinger and Holzapfel 1990; Schillinger *et al.* 1991; Spelhaug and Harlander 1989). This approach has been demonstrated (to varying degrees of success) to be of potential value in controlling the growth of the foodborne pathogen in dairy products and meats (Beliard *et al.* 1989; Berry *et al.* 1990; Chung *et al.* 1989; Gouet *et al.* 1978; Nielsen *et al.* 1990; Pucci *et al.* 1988; Schillinger *et al.* 1991; Yousef *et al.* 1991). Recently, we (Buchanan and Klawitter 1990) observed the unexpected inactivation of *L. monocytogenes* in a raw ground beef sample, and subsequently isolated a lactic acid bacterium that was antagonistic to the pathogen. The lactic acid bacterium, which had been tentatively identified as *Lactobacillus plantarum*, appeared to be most active at refrigeration temperatures; a characteristic that offers distinct advantages for controlling the growth of *L. monocytogenes* in foods. Accordingly, the objectives of the current study were to (1) investigate the mode of action underlying the isolate's antagonistic activity and (2) characterize cultural conditions that might affect the effectiveness of the lactic acid bacterium against *L. monocytogenes*.

MATERIALS AND METHODS

Microorganisms

Lactic acid bacterium LK5 (initially classified as *Lactobacillus plantarum* LK5) (Buchanan and Klawitter 1990) and *Listeria monocytogenes* Scott A were maintained in Tryptic Soy Broth with Glucose (0.25%) (TSB+G) (Difco Laboratories, Inc., Detroit, MI) stored at 4°C, and transferred periodically. Starter cultures of LK5 and Scott A were grown in Tryptic Soy Broth without Glucose (TSB-G) (Difco) on a rotary shaker (150 rpm) for 24 h at 28°C. Additional *Listeria* strains used for screening purposes are listed in Table 1, and were maintained in the same manner as Scott A.

TABLE 1.
LISTERIA ISOLATES DISPLAYING ZONES OF INHIBITION WHEN GROWN IN
 CONJUNCTION WITH *CARNOBACTERIUM PISCICOLA* LK5

Species	Strain	Source	Inhibited by LK5
<i>L. monocytogenes</i>	Scott A	Clinical, FDA	+
	F-4243	CDC	+
	V37	Milk, FDA	+
	F2-VJ-G	Fish, USDA	+
	CCR8-V-G	Crab, USDA	+
	MF2-L-P	Fish, USDA	+
	HO-VJ-S	Beef, USDA	+
	GVN4-V-G	Veal, USDA	+
	Brie-1	Cheese, FDA	+
	V7	Milk, FDA	-
Murray B	Clinical, FDA	-	
<i>L. innocua</i>	GV9-L-S	Veal, USDA	+
	SH3-VJ	Shellfish, USDA	+
	LA-1	CDC	-
	SA3-V-T	Salami, USDA	-
<i>L. welshimeri</i>	GVL4-L-S	Veal, USDA	+
	CCR6-L-G	Crab, USDA	+
	CF-L-P	Shellfish, USDA	+
<i>L. ivanovii</i>	KCI-1714	CDC	+
	F-6000	CDC	+
<i>L. grayii</i>	F-4085	CDC	+

Reagents

Catalase, trypsin, papain, and chymopapain were obtained from Sigma Chemical Co. (St. Louis, MO), and pepsin from Worthington Diagnostic Systems, Inc. (Freehold, NJ). All other chemicals were reagent grade.

Seeded Lawn Assays

Brain Heart Infusion Agar (BHIA) (Difco) spread plates were prepared with 200 μL of a 24 h *L. monocytogenes* Scott A culture. After drying, 5 μL of a 24 h culture of LK5 was spotted in the center of the plate. The plates were incubated for 24 h at 28C and then examined for zones of inhibition.

The procedure was modified to determine the effect of catalase on the activity of LK5 by incorporating sufficient enzyme to achieve a final activity of 100 units/mL. The catalase was added as a filter sterilized solution to BHIA after autoclaving and equilibrating to 50C. The effects of proteolytic enzymes on the activity of LK5 were tested by preparing 10 mg/mL and 30 mg/mL solutions of pepsin, trypsin, papain, and chymopapain in 0.1 M sodium phosphate buffer (pH 7.0). The solutions were sterilized by filtration. Sterile 1/4 in. analytical disks were dipped in the appropriate solution and placed 1.5 cm away from the LK5 inoculum on the BHIA plates seeded with a lawn of Scott A.

Liquid Co-Cultures

After making any desired adjustments to pH (using 10N HCl or 50% NaOH), NaCl content, etc., 100 mL portions of TSB-G were dispensed in 250-mL Erlenmeyer flasks, sealed with foam plugs, and sterilized by autoclaving for 15 min at 121C. The flasks were inoculated with 0.1 mL each of dilutions of 24 h cultures of LK5 and Scott A. Unless otherwise specified, the target inoculum for each microorganism was 10^3 cfu/mL. Flasks inoculated with only *L. monocytogenes* served as controls. Anaerobic cultures were grown in a similar manner using 250-mL trypsinizing flasks according to the technique described previously (Buchanan *et al.* 1989). After inoculation, each flask was sealed with a rubber stopper and a rubber septum, and flushed with nitrogen for 15 min. This was sufficient to reduce the oxygen level to approximately 100–125 ppm. All flasks were incubated on rotary shakers (150 rpm) at 5 or 19C.

Periodically, 5.0 mL samples were removed from each flask, diluted appropriately with sterile 0.1% peptone water, and plated in duplicate using a Spiral Plater (Model D, Spiral Systems, Inc., Bethesda, MD) on pre-poured BHIA and Vogel Johnson agar (VJA) (Difco) plates. The BHIA plates, which were used to provide a total aerobic plate count (TAPC) of the bacteria in the co-cultures (LK5 + Scott A), were incubated for 24 h at 28C. The VJA plates, on which the LK5 did not grow but on which *L. monocytogenes* produces characteristic black colonies, were incubated for 48 h at 37C. The level of LK5 could be estimated by subtracting the VJA value from the BHIA count.

Cell-Free Culture Supernatants

Cell-free culture supernatants were prepared by inoculating sterile 250-mL Erlenmeyer flasks containing 100 mL of Brain Heart Infusion Broth (Difco) with 1.0 mL of a 24 h culture of LK5. The flasks were incubated at 5C (168 and 240 h), 12C (72 and 168 h), 19C (48 and 96 h), and 28C (24 and 72 h) on rotary shakers (150 rpm). The incubation times were selected to provide cultures in the late exponential and stationary growth phases. The cultures were centrifuged to remove the bulk of the cells, followed by filtration through a 0.22 μ m filter. The supernatants were then freeze-dried, and rehydrated in 6.0 mL of 0.1 M phosphate buffer (pH 6.5). Additional cell-free culture supernatants were produced in a similar manner using 5 or 19C cultures, and were either used directly or concentrated by freeze-drying.

The relative activity of the cell-free extracts was estimated using a modification of the seeded lawn assay. A series of 2-fold dilutions were prepared using 0.1M phosphate buffer (pH 6.5) and transferred in 25 μ L portions to sterile disks that had been applied to the surface of duplicate BHIA plates that had been previously seeded with *L. monocytogenes* Scott A. After incubation for 24 h at 28C, the plates were observed for zones of inhibition, with the highest dilution producing a distinct zone > 2 mm being recorded. The activity of cell-free extracts were also assessed by transferring 0.1 mL quantities to wells cut in seeded plates.

RESULTS

Identity of Isolate

The isolate, LK5, had been originally identified as *Lactobacillus plantarum* based on criteria outlined in *Bergey's Manual* (Buchanan and Klawitter 1990). However, there were several characteristics that were inconsistent with this initial identification, particularly the isolate's ability to grow rapidly at 5C and its inability to grow at a pH of 4.5. Recently, Collins *et al.* (1987) proposed a new genus, *Carnobacterium*, to differentiate a group of atypical lactic acid bacteria isolated from refrigerated meat and poultry. Among their characteristics, these bacteria were catalase-negative, oxidase-negative, and unable to grow on acetate medium or in the presence of 8% NaCl. Members of the genus grow at refrigeration temperatures but not at 45C. The current isolate has similar characteristics including being unable to grow on Rogosa agar. These characteristics indicated that LK5 is a member of the genus *Carnobacterium* instead of *Lactobacillus*. Comparison of the isolate's characteristics, particularly its fatty acid profile (Table 2) and sugar fermentations (Buchanan and Klawitter

TABLE 2.
COMPARISON OF THE FATTY ACID PROFILE OF *CARNOBACTERIUM PISCICOLA* LK5 TO
THAT OF REFERENCE STRAINS

Fatty Acid	% of Total	
	LK5 ^a	Reference ^b
14:0	13.2	10.0 - 11.0
16:0	15.6	14.0 - 18.5
16:1	15.4	11.0 - 16.0
18:0	2.0	1.5 - 3.5
18:1 cis 9	49.4	49.5 - 53.5

^a Values provided by Microchek, Inc.

^b Ranges reported by Collins et al. (1987) for six strains of *C. piscicola*.

1990), against the speciation scheme proposed by Collins *et al.* (1987) is consistent with the identification of LK5 as *Carnobacterium piscicola*, and it will be referred to accordingly.

Characterization of Antilisterial Activity

Using a seeded lawn technique, LK5 was screened against 21 strains of *Listeria* which included five of the genus' species (Table 1). Distinct zones of inhibition were evident with seventeen of the strains. *L. monocytogenes* Scott A, which gave a distinct positive response, was used for all subsequent experimentation. No activity was noted when LK5 was screened against a variety of other Gram-positive and Gram-negative species including *Staphylococcus aureus* (196E, NRRL B-121, NRRL B-124, and NRRL B-767), *Bacillus cereus*

(T and H-13), *Bacillus thuringiensis* (E-2), *Vibrio parahaemolyticus* (V10 and V12), *Vibrio vulnificus* (V3 and V7), *Escherichia coli* (933, A9218-C1, and 4575-35), *Shigella dysenteriae* (20011), *Shigella boydii* (20032), *Shigella sonnei* (20009), *Yersinia enterocolitica* (GER), *Salmonella typhimurium* (Thax-1 and TML-R66), *Salmonella dublin* (15480), *Salmonella enteritidis* (2000), and *Aeromonas hydrophila* (K114).

The role that acid formation played in relation to the inhibitory response was assessed by co-culturing Scott A and LK5 at 5 and 19C in TSB-G and TSB+G (Fig. 1). The inhibitory response in liquid co-cultures was distinctly different from that observed with seeded lawns assays. When cell-free supernatants from LK5 cultures were added to 24-h Scott A cultures (approximately 10^9 cfu/mL), bactericidal activity was evident within 5 min (data not shown).

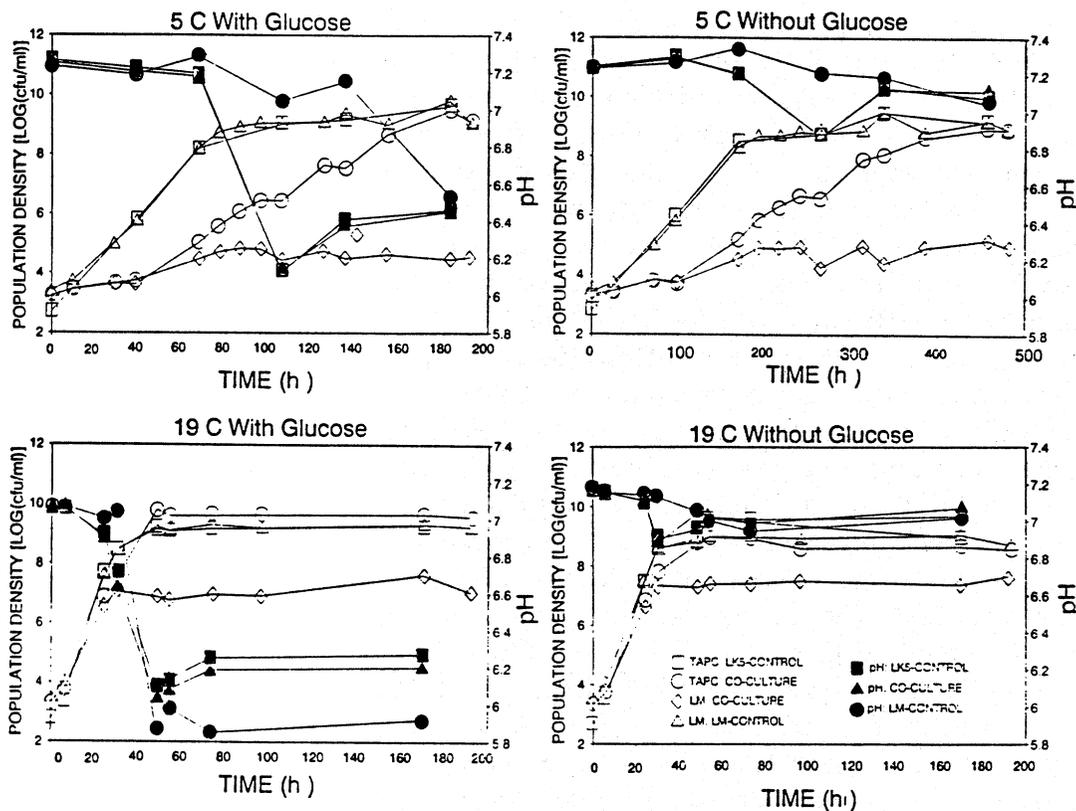


FIG. 1. ABILITY OF *CARNOBACTERIUM PISCICOLA* LK5 TO INHIBIT THE GROWTH OF *LISTERIA MONOCYTOGENES* SCOTT A AT 5 AND 19C IN TRYPTIC SOY BROTH WITH AND WITHOUT GLUCOSE

LK5 - Control and TAPC (Co-culture): = counts (*Carnobacterium* + *Listeria*) using Brain Heart Infusion agar. LM: = *L. monocytogenes* counts (co-culture and control) using Vogel Johnson agar. Values are means of six replicates assayed in duplicate.

However, when liquid media were inoculated with low levels of the two microorganisms, bacteriostasis, as evidenced by a distinct suppression of the maximum population density achieved by the pathogen, was the primary effect. This suppression of *L. monocytogenes* typically coincided with attainment of stationary growth by LK5, suggesting that the synthesis of the active inhibitory agent occurs during the late logarithmic phase of growth. The response observed was temperature dependent, with a substantially greater suppression of *L. monocytogenes* occurring in the 5C co-cultures. Part of this differential response may have been a function of the *C. piscicola* isolate being capable of substantially faster growth than *L. monocytogenes* at refrigeration temperatures, whereas their growth rates were approximately equivalent at 19C. This would result in LK5 attaining late logarithmic growth relatively earlier in relation to the growth cycle of Scott A when co-cultures were incubated at 5C. No difference in antilisterial activity was observed with glucose-containing and glucose-free media despite differences in final pH values. Even with the glucose-containing medium, the extent of pH decline was small enough such that it would not be expected to produce a substantive inhibition of *L. monocytogenes* growth (Buchanan *et al.* 1989). No loss of activity was observed when cell-free supernatants were adjusted to pH 6.5. These results indicated that the antagonistic activity of LK5 is not dependent on acid production.

Lactic acid bacteria have been shown to inhibit the growth of other bacteria via the formation of peroxides, particularly hydrogen peroxide, even when the effected species synthesizes catalase (Beliard *et al.* 1989). This possibility was evaluated by seeded lawn assay using BHIA with and without 100 units/mL of catalase. Catalase supplementation did not affect the zones of inhibition observed on the plates. Pieces of BHIA excised from the zones of inhibition on the catalase-containing plates were confirmed qualitatively as having high levels of enzyme activity at the end of the assay. While these results did not rule out the possible synthesis of inhibitory organic peroxides, they do indicate that LK5's mode of action does not involve the formation of hydrogen peroxide.

As indicated earlier, a number of the bacteria with activity against *L. monocytogenes* have been found to produce bacteriocins (Ahn and Stiles 1990; Berry *et al.* 1990; Bhunia *et al.* 1988; Carminati *et al.* 1989; Harris *et al.* 1989; Hoover *et al.* 1988, 1989; Lewus *et al.* 1991; Ortel 1989; Pucci *et al.* 1988; Schillinger and Lucke 1989; Schillinger and Holzapfel 1990; Spelhaug and Harlander 1989). This possible means of inhibition was assessed using a seeded lawn which an absorbant disk impregnated with one of four proteolytic enzymes (trypsin, pepsin, papain, and chymopapain) was placed in proximity of the LK5 inoculum. Trypsin, papain, and to a lesser extent chymopapain eliminated the activity of LK5 against *L. monocytogenes*, whereas pepsin was ineffective. Similar results were obtained when cell-free culture supernatants were

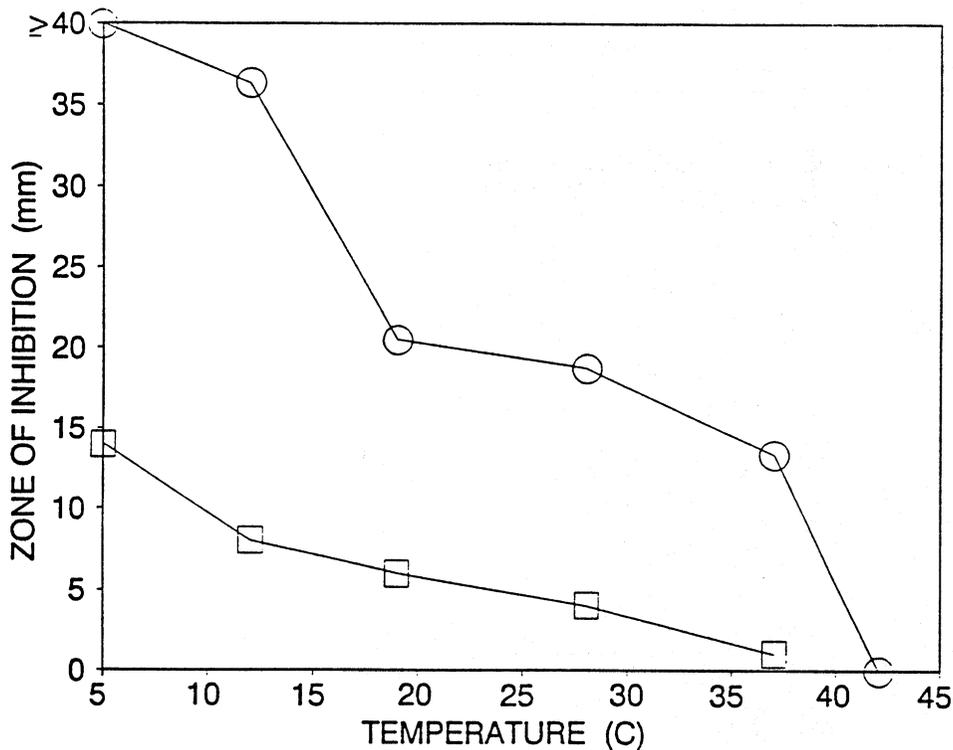


FIG. 2. EFFECT OF INCUBATION TEMPERATURE ON THE ACTIVITY OF *CARNOBACTERIUM PISCICOLA* LK5 CULTURES AND CELL-FREE EXTRACT

□ Size of zones of inhibition produced when BHIA plates seeded with *Listeria monocytogenes* Scott A were inoculated with *C. piscicola* LK5 and incubated at various temperatures.

○ Size of zones of inhibition produced when 0.1 mL of a cell-free extract of a 19C culture of *C. piscicola* LK5 was added to wells in BHIA plates seeded with *L. monocytogenes* Scott A and then incubated at different temperatures.

treated with the proteolytic enzymes. The loss of activity following protease treatment was indicative of a bacteriocin.

The temperature dependent production of the bacteriocin was examined further using plates seeded with a lawn of *L. monocytogenes*, point inoculated with LK5, and incubated at 5, 12, 19, 28, and 37C. The size of the zones of inhibition observed was inversely related to the incubation temperature (Fig. 2), indicating that either the synthesis of the apparent bacteriocin or the susceptibility of *L. monocytogenes* was temperature dependent. Adding a 0.1 mL aliquots of cell-free supernatant from a 19C LK5 culture to wells cut in seeded plates and then incubating the plates at various temperatures indicated that the sensitivity of *L. monocytogenes* to the bacteriocin increased at lower incubation temperatures (Fig. 2). Titration of cell-free supernatants prepared from late exponential and stationary phase LK5 grown at 5, 12, 19, and 28C (data not shown), indicated that bacteriocin production was roughly equivalent at the

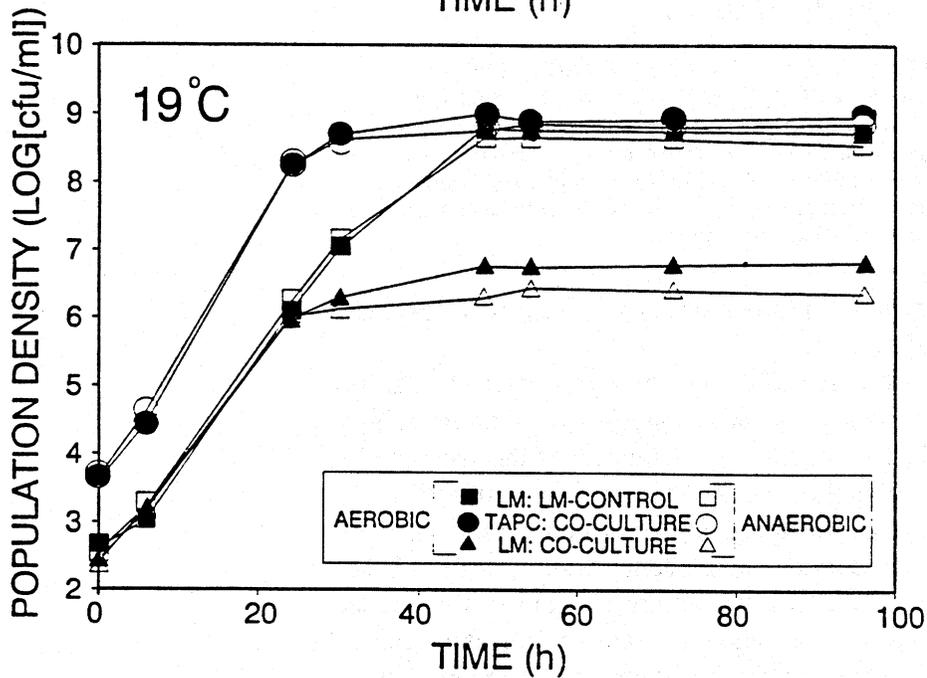
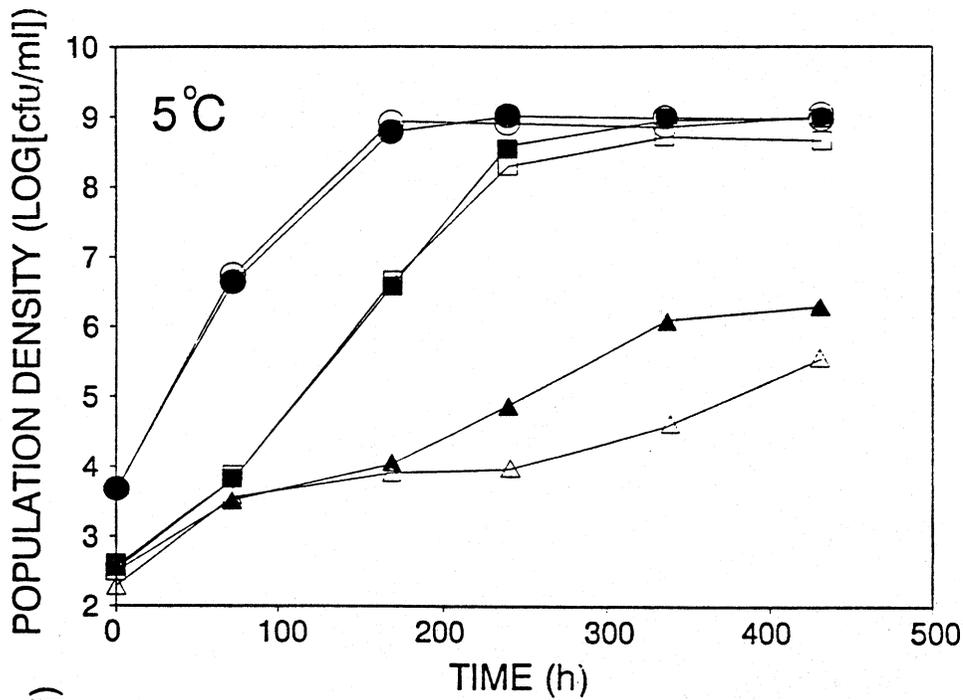


FIG. 3. EFFECT OF OXYGEN AVAILABILITY ON THE ABILITY OF *CARNOBACTERIUM PISCICOLA* LK5 TO SUPPRESS THE GROWTH OF *LISTERIA MONOCYTOGENES* SCOTT A WHEN CO-CULTURED IN TRYPTIC SOY BROTH (WITHOUT GLUCOSE) AT 5 AND 19°C. Values are means of two replicates assayed in duplicate.

three lower temperatures, but was reduced substantially in the 28C cultures. Activity levels were somewhat higher in the late exponential phase cultures. The overall level of activity produced in liquid cultures was low and in some instances the cell-free supernatants had to be concentrated 10-fold to detect the bacteriocin in assays using absorbant disks. It would appear that the temperature dependent nature of LK5 activity is primarily a function of *L. monocytogenes* susceptibility, but is also influenced by decreased bacteriocin production at higher temperatures.

The thermal stability of the active agent was assessed by heating a cell-free supernatant from a 5C culture at 60, 80, and 100C for 5 min. The agent produced by LK5 was quite heat resistant with no loss of activity observed with the 60 and 80C treatments, and less than a 50% loss in activity with the 100C heating.

Effect of Cultural Conditions

The ability of the *Carnobacterium* isolate to grow and produce a bacteriocin at 5C suggested that it could be used to competitively suppress *L. monocytogenes* in refrigerated foods. This possibility was assessed further by determining in microbiological media how various cultural parameters commonly

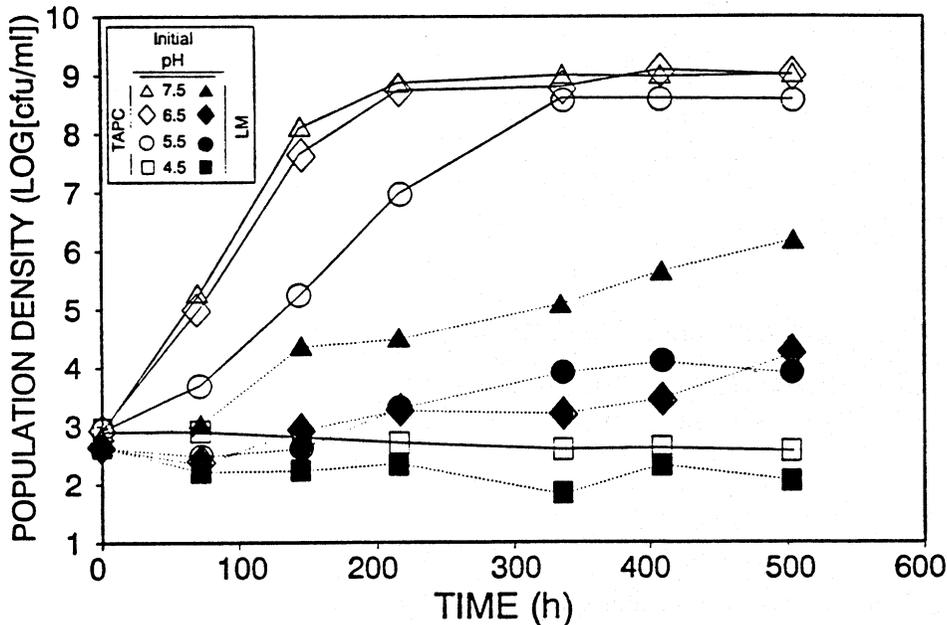


FIG. 4. EFFECT OF INITIAL pH ON THE ABILITY OF *CARNOBACTERIUM PISCICOLA* LK5 TO SUPPRESS THE GROWTH OF *LISTERIA MONOCYTOGENES* SCOTT A WHEN CO-CULTURED IN TRYPTIC SOY BROTH (WITHOUT GLUCOSE) AT 5 AND 19C. Values are means of two replicates assayed in duplicate.

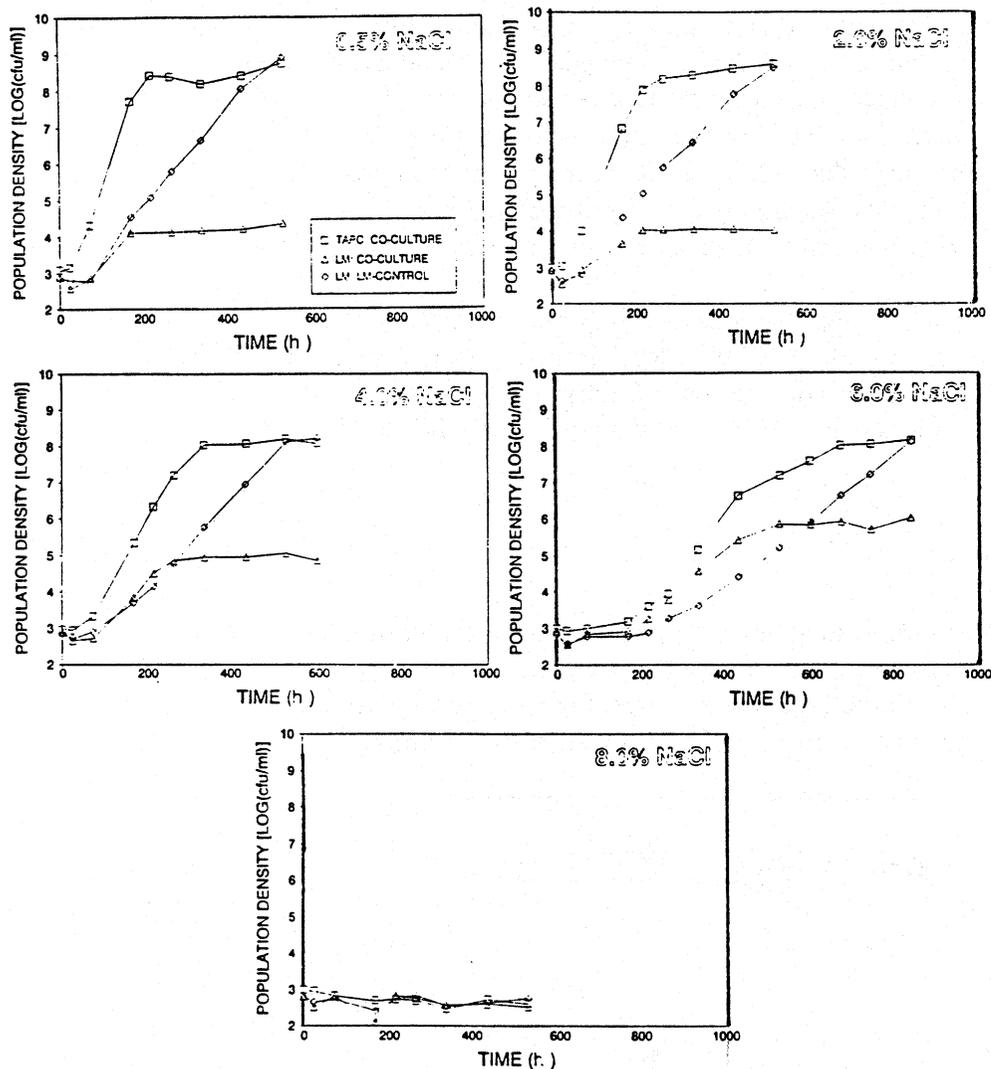


FIG. 5. EFFECT OF NA₂CL CONTENT ON THE ABILITY OF CARNOBACTERIUM PISCICOLA LK5 TO SUPPRESS THE GROWTH OF LISTERIA MONOCYTOGENES SCOTT A WHEN CO-CULTURED IN TRYPTIC SOY BROTH (WITHOUT GLUCOSE) AT 5C. Values are means of two replicates assayed in duplicate.

affecting microbial growth in food systems would likely impact the effectiveness of LK5. The effect of restricting oxygen availability was determined by comparing aerated versus nitrogen-flushed, sealed co-cultures (Fig. 3). At 19C, there was little differential in antilisterial activity between the aerobic and anaerobic cultures. At 5C, a greater suppression of *L. monocytogenes* was transi-

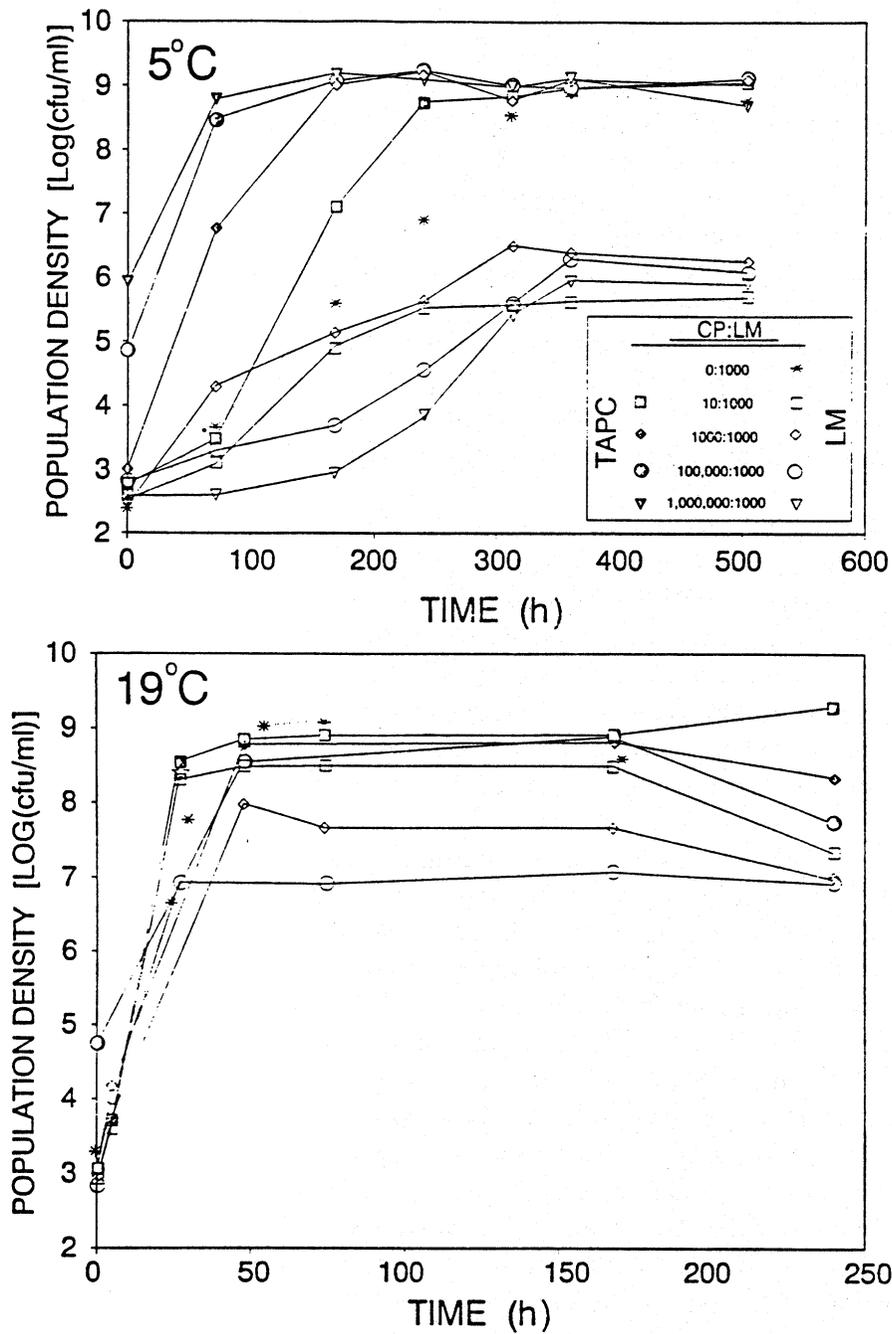


FIG. 6. EFFECT OF INOCULUM LEVEL RATIO OF *CARNOBACTERIUM PISCICOLA* LKS TO *LISTERIA MONOCYTOGENES* SCOTT A ON THE SUBSEQUENT ABILITY OF THE LACTIC ACID BACTERIUM TO SUPPRESS THE GROWTH OF THE PATHOGEN WHEN CO-CULTURED IN TRYPTIC SOY BROTH (WITHOUT GLUCOSE) AT 5 AND 19C. Values are means of two replicates assayed in duplicate. Values for CP:LM ratios of 100:1000 and 10,000:1000 were omitted for the sake of clarity. They have responses intermediate to those depicted. A CP:LM ratio of 1,000,000:1000 was not performed at 19C.

torily observed in the anaerobic cultures. However, by the end of the incubation period, the antilisterial activity of LK5 was roughly equivalent in the aerobic and anaerobic co-cultures. These results indicate that the efficacy of LK5 would not be adversely affected by restricting oxygen levels.

Examination of the effect of initial pH on the growth of *L. monocytogenes* at 5C in the presence of LK5 indicated that the bacterium was most effective at pH values of 5.5 and 6.5 (Fig. 4). The pathogen attained somewhat higher levels when the initial pH was 7.5. Neither microorganism was capable of growth at refrigerated temperatures within 500 h when the initial pH was 4.5. It appears that LK5 would be effective over the pH range of concern in relation to the growth of *L. monocytogenes*, though its efficacy would be greater if the pH was below 7.0.

The antilisterial activity of LK5 at 5C was unaffected when the NaCl content of TSB-G was increased to 2% (Fig. 5). Increasing the NaCl content to 4% depressed the activity of LK5 somewhat, with a substantially greater loss of activity being evident at 6% NaCl. The growth of both *L. monocytogenes* and *C. piscicola* were delayed at 6% NaCl, and neither species grew at 8% NaCl. These data indicated that LK5 would be active against *L. monocytogenes* over the salt levels of concern in relation to the growth of the pathogen at refrigerated temperatures, with the lactic acid bacterium being most effective at NaCl concentrations $\leq 4\%$.

The relative competitiveness of LK5 was evaluated by determining the effect of inoculum ratio on the ability of LK5 to suppress the growth of *L. monocytogenes* (Fig. 6). The initial level of the pathogen was kept constant at approximately 1000 cfu/mL, while the inoculum level of *Carnobacterium* was varied in 10-fold increments over the range from 10 cfu/mL to 1,000,000 cfu/mL. At 19C, the degree of suppression of *L. monocytogenes* was dependent on inoculum ratio, with only LK5:Scott A ratios $\geq 1:1$ producing any substantial degree of inhibition. At 5C, an increased level of antilisterial activity was observed with the higher inoculum ratios during the early stages of the incubation, presumably due to bacteriocin accumulating earlier in these cultures. However, by approximately 300 h of incubation when *L. monocytogenes* levels stabilized, LK5 inoculum size had no effect on inhibitory activity; the degree of suppression was equivalent for LK5:Scott A ratios ranging from 0.01:1 to 1000:1. The *Carnobacterium* isolate was extremely competitive at refrigeration conditions, indicating that even small inocula could be used to control *L. monocytogenes* in refrigerated foods.

DISCUSSION

The ability of bacteria to inhibit the growth of *L. monocytogenes* appears to be related to specific characteristics associated with certain competitors rather than the simple attainment of high bacterial numbers (Mattila-Sandholm and Skytla 1991; Tran *et al.* 1990). This was aptly demonstrated in the current study by the temperature dependent nature of LK5's inhibitory activity. Even when overwhelmingly large inocula were used (Fig., 6), activity against the pathogen was greatly reduced at elevated incubation temperatures, despite the maximum population density attained by LK5 being largely independent of incubation temperature. Potentially, there are a number of means by which a lactic acid bacterium could inhibit the growth of *L. monocytogenes*, including most obviously, the production of acid. While the current isolate can produce acid from a variety of sugars (Buchanan and Klawitter 1990), neither its growth nor its antilisterial activity is dependent on the presence of a fermentable carbohydrate (Fig. 1). This is not surprising considering the strain was originally isolated from a refrigerated raw ground beef sample that had a pH of 7.2–7.4 (Buchanan and Klawitter 1990). Instead, like a number of other lactic acid bacteria with antilisterial activity (Ahn and Stiles 1990; Berry *et al.* 1990; Bhunia *et al.* 1988; Carminati *et al.* 1989; Gouet *et al.* 1978; Harris *et al.* 1989; Hoover *et al.* 1988, 1989; Lewus *et al.* 1989; Ortel 1989; Pucci *et al.* 1988; Schillinger and Lucke 1989; Schillinger and Holzapfel 1990; Spelhaug and Harlander 1989), the suppression of *Listeria* by LK5 appears to be related to the synthesis of a bacteriocin.

Several groups of investigators have recently reported the identification of *Carnobacterium* isolates that produce bacteriocins against *L. monocytogenes*. Ahn and Stiles (1990) reported that *C. piscicola* LV17 produced two bacteriocins. They hypothesized that the bacteriocins were associated with the presence of 40- and 49-Mdl plasmids. Lewus *et al.* (1991) isolated two strains of *C. piscicola* with activity against multiple strains of *L. monocytogenes*. Schillinger and Holzapfel (1990) observed that 18 of 37 isolates of *Carnobacterium* spp. had activity against a *L. monocytogenes* isolate using a spot test with a viable culture. Activity was particularly prevalent with *C. piscicola* [10 of 13 strains including strain LV17 studied by Ahn and Stiles (1990)]. However, none of the isolates had activity when cell-free supernatants were assayed using a well diffusion test. Schillinger and Holzapfel (1990) attributed this difference to the localized concentration associated with the spot test versus the lower concentrations achieved with liquid cultures. They reported that concentrating the cell-free supernatant of *C. piscicola* LV61 10-fold gave a positive well diffusion test. The relationship between the bacteriocins observed by the above investigators and LK5 awaits future comparisons. Preliminary results indicate

that LK5 does not contain a plasmid, and studies examining the genetics of bacteriocin synthesis, as well as the purification of the agent, are underway.

Bacteriocins are generally considered to have a bactericidal mode of action, and the antimicrobial agent produced by LK5 fits this criterion when used in assays that provide a localized high concentration of the bacteriocin. However, when co-cultures were employed, the observed response was indicative of bacteriostasis. The reason for this differential will require further investigation. It may be a function of the concentration of bacteriocin synthesized by LK5 in liquid culture as compared to on agar plates. This would be similar to the observations of Schillinger and Holzapfel (1990) and Harris *et al.* (1989). Part of the difference may also be due to the low inoculum levels employed in the current study. Most other investigations have employed extremely large inocula, whereas the current study was designed to deal with levels that might actually occur in food products. Finally, while there are reports where liquid co-cultures have produced clearly bactericidal responses (Schillinger and Lucke 1989; Yousef *et al.* 1991), the observation that bacteriocins can be bactericidal or bacteriostatic in different conditions is not without precedent. For example, Schillinger *et al.* (1991) reported that *Lactobacillus sake* was bactericidal to *L. monocytogenes* in MRS broth, but was bacteriostatic in minced meat. Further, it has been noted by several investigators that after an initial exposure to a bacteriocin produces a bactericidal response, the surviving cells may remain in a state of stasis for extended periods (Berry *et al.* 1990; Bhunia *et al.* 1988; Nielsen *et al.* 1990).

There are two primary means by which the activity of LK5 could be used to help control *L. monocytogenes*. The bacteriocin could be isolated and used as an additive to food systems as currently being done with nisin. While this approach has a number of advantages such as ease of control and minimizing organoleptic changes, it has several disadvantages such as cost, availability, and possible requirement for approval as a food additive. For these reasons, this initial work concentrated on the feasibility of utilizing *C. piscicola* LK5 directly as a starter culture. Based on a model systems evaluation of a variety of food formulation and storage factors that would be likely to influence the bacterium, it appears that the microorganism would be most effective against *L. monocytogenes* in refrigerated products, and would be less useful in nonrefrigerated or temperature abused products. Within that constraint, it appears that LK5 would be effective in a variety of products. This includes being effective over a range of water activities as demonstrated by its NaCl tolerance (Fig. 5), having activity over the pH ranges of concern with *L. monocytogenes* (Fig. 4), and being equally active under aerobic and oxygen restricted conditions (Fig. 3). Further, its highly competitive growth at refrigeration temperatures permits the use of extremely low inocula (Fig. 6), suggesting that this would be a high-

ly efficient process. It appears that the direct incorporation of *C. piscicola* LK5 has significant potential as a means of controlling the growth of *Listeria* in refrigerated products. This supposition requires confirmation using a range of food products, including determining the impact of the lactic acid bacterium on organoleptic characteristics. An initial assessment of LK5's effectiveness in a number of foods has been completed and will be reported separately.

ACKNOWLEDGMENTS

We would like to gratefully acknowledge Microchek, Inc. (P.O. Box 456, 48 South Main Street, Northfield, VT 05663) for graciously performing the fatty acid profile of the *C. piscicola* isolate.

REFERENCES

- AHN, C. and STILES, M.E. 1990. Plasmid-associated bacteriocin production by a strain of *Carnobacterium piscicola* from meat. *Appl. Environ. Microbiol.* 56, 2503–2510.
- BELIARD, E., ABGRALL, B. and BOURGEOIS, C.M. 1989. Selection de souches de bacteries lactiques inhibitrices de *Pseudomonas putida*. *Sci. Aliments* 9, 665–684.
- BENKERROUM, N. and SANDINE, W.E. 1988. Inhibitory action of nisin against *Listeria monocytogenes*. *J. Dairy Sci.* 71, 3237–3245.
- BERRY, E.D., LIEWEN, M.B., MANDIGO, R.W. and HUTKINS, R.W. 1990. Inhibition of *Listeria monocytogenes* by bacteriocin-producing *Pediococcus* during the manufacture of fermented semidry sausage. *J. Food Protect.* 53, 194–197.
- BHUNIA, A.K., JOHNSON, M.C. and RAY, B. 1988. Purification, characterization and antimicrobial spectrum of a bacteriocin produced by *Pediococcus acidilactici*. *J. Appl. Bacteriol.* 65, 261–268.
- BUCHANAN, R.L. and KLAWITTER, L.A. 1990. Effect of temperature history on the growth of *Listeria monocytogenes* Scott A at refrigeration temperatures. *Int. J. Food Microbiol.* 12, 235–245.
- BUCHANAN, R.L., STAHL, H.G. and WHITING, R.C. 1989. Effects and interactions of temperature, pH, atmosphere, sodium chloride, and sodium nitrite on the growth of *Listeria monocytogenes*. *J. Food Protect.* 52, 844–851.
- CARMINATI, D., GIRAFFA, G. and BOSSI, M.G. 1989. Bacteriocin-like inhibitors of *Streptococcus lactis* against *Listeria monocytogenes*. *J. Food Protect.* 52, 614–617.

- CHUNG, K.-T., DICKSON, J.S. and CROUSE, J.D. 1989. Effects of nisin on growth of bacteria attached to meat. *Appl. Environ. Microbiol.* 55, 1329–1333.
- COLLINS, M.D., FARROW, J.A.E., PHILLIPS, B.A., FERSUSU, S. and JONES, D. 1987. Classification of *Lactobacillus divergens*, *Lactobacillus piscicola*, and some catalase-negative, asporogenous, rod-shaped bacteria from poultry in a new genus, *Carnobacterium*. *Int. J. Sys. Bacteriol.* 37, 310–316.
- GOUET, P., LABADIE, J. and SERRATORE, C. 1978. Development of *Listeria monocytogenes* in monoxenic and polyxenic beef minces. *Zbl. Bakteriolog. Hyg. Abt. Orig. B.* 166, 87–94.
- HARRIS, L.J., DAESCHEL, M.A., STILES, M.E. and KLAENHAMMER, T.R. 1989. Antimicrobial activity of lactic acid bacteria against *Listeria monocytogenes*. *J. Food Protect.* 52, 384–387.
- HOOVER, D.G., DISHART, K.J. and HERMES, M.A. 1989. Antagonistic effect of *Pediococcus* spp. against *Listeria monocytogenes*. *Food Biotechnol.* 3, 183–196.
- HOOVER, D.G., WALSH, P.M., KOLAETIS, K.M. and DALY, M.M. 1988. A bacteriocin produced by *Pediococcus* species associated with a 5.5-megadalton plasmid. *J. Food Protect.* 51, 29–31.
- LEWUS, C.B., KAISER, A. and MONTVILLE, T.J. 1991. Inhibition of food-borne bacterial pathogens by bacteriocins from lactic and bacteria isolated from meat. *Appl. Environ. Microbiol.* 57, 1683–1688.
- MATTILA-SANDHOLM, T. and SKYTLA, E. 1991. The effect of spoilage flora on the growth of food pathogens in minced meat stored at chilled temperatures. *Lebensm.-Wiss. Technol.* 24, 116–120.
- NIELSEN, J.W., DICKSON, J.S. and CROUSE, J.D. 1990. Use of a bacteriocin produced by *Pediococcus acidilactici* to inhibit *Listeria monocytogenes* associated with fresh meat. *Appl. Environ. Microbiol.* 56, 2142–2145.
- ORTEL, S. 1989. Listeriocins (monocins). *Int. J. Food Microbiol.* 8, 249–250.
- PUCCI, M.J., VEDAMUTHU, E.R., KUNKA, B.S. and VANDENBERGH, P.A. 1988. Inhibition of *Listeria monocytogenes* by using bacteriocin PA-1 produced by *Pediococcus acidilactici* PAC 1.0. *Appl. Environ. Microbiol.* 54, 2349–2353.
- SCHILLINGER, U. and HOLZAPFEL, W.H. 1990. Antibacterial activity of carnobacteria. *Food Microbiol.* 7, 305–310.
- SCHILLINGER, U., KAYCE, M. and LUCKE, F.-K. 1991. Behavior of *Listeria monocytogenes* in meat and its control by a bacteriocin-producing strain of *Lactobacillus sake*. *J. Appl. Biol.* 70, 473–476.
- SCHILLINGER, U. and LUCKE, F.-K. 1989. Antibacterial activity of *Lactobacillus sake* isolated from meat. *Appl. Environ. Microbiol.* 55, 1901–1906.

- SPELHAUG, S.R. and HARLANDER, S.K. 1989. Inhibition of foodborne bacterial pathogens by bacteriocins from *Lactococcus lactis* and *Pediococcus pentosaceus*. *J. Food Protect.* 52, 856–862.
- TRAN, T.T., STEPHENSON, P. and HITCHINS, A.D. 1990. The effect of aerobic mesophilic microfloral levels on the isolation of inoculated *Listeria monocytogenes* strain LM82 from selected foods. *J. Food Safety* 10, 267–275.
- YOUSEF, A.E., LUCHANSKY, J.B., DEGNAN, A.J. and DOYLE, M.P. 1991. Behavior of *Listeria monocytogenes* in wiener exudates in the presence of *Pediococcus acidilactici* H or pediocin AcH during storage at 4 or 25°C. *Appl. Environ. Microbiol.* 57, 1461–1467.