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**EFFECTIVENESS OF *CARNOBACTERIUM PISCICOLA* LK5
FOR CONTROLLING THE GROWTH OF *LISTERIA*
MONOCYTOGENES SCOTT A IN REFRIGERATED FOODS¹**

ABSTRACT

The potential for controlling the growth of Listeria monocytogenes in refrigerated foods using Carnobacterium piscicola LK5, a bacteriocin-producing strain originally isolated from raw ground beef, was studied using co-culture techniques. Eight foods, including UHT milk, canned "all-beef" dog food (cooked meat), raw ground beef, irradiation-sterilized raw ground beef, chicken roll, pasteurized crabmeat, canned creamed corn, and frankfurters, were inoculated with 10³ cfu/g L. monocytogenes Scott A, with and without 10⁴ cfu/g LK5, and incubated at 5 and 19C. Samples were removed periodically and assayed for total aerobic plate count using Brain Heart Infusion Agar and L. monocytogenes using Vogel-Johnson Agar or Modified Vogel Johnson Agar. The growth of L. monocytogenes was suppressed in milk, dog food, crabmeat, creamed corn, and frankfurters stored at 5C. The microorganism was less inhibitory at 19C. In sterile raw ground beef, LK5 inactivated the pathogen at 5C and prevented its growth at 19C. No activity attributable to LK5 was observed in refrigerated nonsterile ground beef or chicken roll; however, these products did not support the psychrotrophic growth of the pathogen even in the absence of LK5. LK5 was most effective in products where the background microflora was reduced by either thermal processing or irradiation treatment. The results indicate that C. piscicola LK5 has potential as a means for preventing the growth of L. monocytogenes in a variety of refrigerated food products.

INTRODUCTION

The need to identify new means for controlling the growth of *Listeria monocytogenes* in refrigerated foods has prompted a number of investigators to identify bacterial species that produce bacteriocins or bacteriocin-like agents with activity against the pathogen (Benkerroum and Sandine 1988; Bhunia *et al.* 1988; Pucci *et al.* 1988; Hoover *et al.* 1988, 1989; Carminati *et al.* 1989; Ortel 1989; Spelhaug and Harlander 1989; Schillinger and Lucke 1989; Harris *et al.* 1989; Ahn and Stiles 1990; Schillinger and Holzapfel 1990; Lewus *et al.* 1991; Buchanan and Klawitter 1991b). While a number of bacteriocin-producing strains have been isolated, there is substantially less information available concerning the efficacy of the microorganisms or their bacteriocins in actual food systems (Pucci *et al.* 1988; Chung *et al.* 1989; Nielsen *et al.* 1990; Berry *et al.* 1990; Schillinger *et al.* 1991; Yousef *et al.* 1991), though these studies do indicate potential applicability. Several groups of investigators have recently reported isolating strains of the newly recognized genus, *Carnobacterium*, that have antilisterial activity (Ahn and Stiles 1990; Lewus *et al.* 1991; Schillinger and Holzapfel 1990), including the isolation of *Carnobacterium piscicola* LK5 in our laboratory (Buchanan and Klawitter 1991a,b). This isolate is highly specific for *L. monocytogenes* and most effective at refrigerated temperatures. This earlier work suggested that the incorporation of either the organism or the isolated bacteriocin could be used to help control *L. monocytogenes* in refrigerated foods (Buchanan and Klawitter 1991b). In the former case, we demonstrated using co-cultures in microbiological systems that *C. piscicola* LK5 was effective at pH, a_w , atmosphere composition, and inoculum levels that would likely be encountered in food systems. The objective of the current study was to expand the use of co-culture techniques to assess the potential for controlling the growth of *L. monocytogenes* Scott A in various refrigerated foods. The foods evaluated included ultra high temperature (UHT) pasteurized milk, raw ground beef (sterile and nonsterile), canned dog food, pasteurized crabmeat, chicken roll, frankfurters, and canned creamed corn.

MATERIALS AND METHODS

Microorganisms

Carnobacterium piscicola LK5, a bacteriocin strain originally isolated from raw ground beef (Buchanan and Klawitter 1991a), and *Listeria monocytogenes* Scott A, a clinical isolate sensitive to the bacteriocin, were used throughout the

study. Working stock cultures were maintained in Brain Heart Infusion Broth (BHI) (Difco Labs, Inc., Detroit, MI) and Tryptic Soy Broth (TSB) (Difco) stored at 4C and subcultured monthly.

Starter Cultures

Starter cultures were initiated by transferring a loopful of the working stocks to 250-mL Erlenmeyer flasks containing 50 mL of sterile Tryptic Soy Broth without Glucose (TSB-G) (Difco). The cultures were incubated on a rotary shaker (150 rpm) for 24 h at 28C.

Culture Techniques

Foods were purchased at a local supermarket on the day an experiment was initiated. The foods were apportioned into three replicates per experimental group and aseptically transferred to sterile containers. The foods, experimental groups, and portion sizes used in the study are summarized in Table 1. The irradiation sterilized raw ground beef was prepared as described by Buchanan and Klawitter (1991a). Prior to being transferred to plastic bags, the frankfurters were immersed in boiling water for 2 min to reduce the microflora.

The target inoculum levels were 10^3 cfu/g for *L. monocytogenes* and 10^4 cfu/g for *C. piscicola*. This was achieved by diluting the starter cultures using sterile 0.1% peptone water, with the final dilution having green food coloring added to the diluent. A 1.0 mL portion of this dilution of the appropriate culture(s) was added to the designated food sample and mixed until the green dye was evenly distributed throughout the food.

A modified procedure was used to surface inoculate the frankfurter samples. Residual bacteriocin in the LK5 starter culture was removed by centrifuging the culture for 10 min at $10,000 \times g$, resuspending the pellet in 15 mL of 0.1 M (pH 7.2) phosphate buffer, recentrifuging, and then suspending the washed cells in 100 mL of 0.1% peptone water. All subsequent operations were carried out under a biological hood. The *L. monocytogenes* culture was transferred in 50 mL portions to two plastic bags. The LK5 culture was also transferred in 50 mL portions to two plastic bags, one of which also contained 50 mL of the *L. monocytogenes* culture. The three plastic bags, which held either LK5, Scott A, or LK5 + Scott A, were brought up to a volume of 600 mL. The frankfurters that had been previously dipped in boiling water were transferred to the appropriate plastic bag and held for 10 min. The frankfurters were then allowed to dry on a paper towel lined tray for 10 min, and transferred to individual plastic bags sealed with a twist tie.

TABLE 1.
THE REPLICATE SIZE, STERILE CONTAINER, EXPERIMENTAL CULTURES EMPLOYED
WITH THE VARIOUS FOODS^a

FOOD	REPLICATE SIZE (g)	STERILE CONTAINER	EXPERIMENTAL CULTURES
Chicken Roll	50	Beaker	LM/LK5 Co-Culture LM-Control LK5-Control
Canned Dog Food	50	Beaker	LM/LK5 Co-Culture LM-Control LK5-Control
Canned Creamed Corn	50	Beaker	LM/LK5 Co-Culture LM-Control LK5-Control
Raw Ground Beef, Non-Sterile	50	Plastic Bag	LM/LK5 Co-Culture LM-Control Uninoculated Control
Raw Ground Beef, Sterile	50	Plastic Bag	LM/LK5 Co-Culture LM-Control Uninoculated Control
UHT Milk	100	Erlenmeyer Flask	LM/LK5 Co-Culture LM-Control
Pasteurized Crabmeat	75	Beaker	LM/LK5 Co-Culture LM-Control Uninoculated Control
Frankfurter	50 Intact Sausage	Plastic Bag	LM/LK5 Co-Culture LM-Control LK5-Control

a. LM - *Listeria monocytogenes* Scott A
LK5 = *Carnobacterium piscicola* LK5

After inoculation, the food samples were incubated at 5 and 19C. Periodically, 1.1 g samples were transferred to screw cap tubes containing 9.9 mL of sterile 0.1% peptone water and agitated for approximately 1 min using a vortex mixer. After making appropriate dilutions, the samples were assayed for total aerobic plate count (TAPC) and *L. monocytogenes* by plating duplicate samples on Brain Heart Infusion Agar (BHIA) (Difco) and one of three forms of Vogel-Johnson Agar, respectively. All plating was performed using a spiral plater (Spiral System Instruments, Inc., Bethesda, MD). The form of Vogel-

Johnson Agar employed was dependent on the type and extent of the competing microflora. Vogel-Johnson Agar (VJA) (Difco) being used with UHT milk, dog food, sterile raw ground beef, and creamed corn; Modified Vogel-Johnson Agar (MVJA) (Buchanan *et al.* 1987) was used with crabmeat and frankfurters; and Modified Vogel-Johnson Agar + 200 µg/mL NaAsO₂ (MVJA + As) (Buchanan and Klawitter 1991a) was employed with nonsterile raw ground beef. The BHIA plates were incubated for 24 h at 28C. The VJA and MVJA plates were incubated at 37C for 48 h, and the MVJA + As for 72 h. The plates were enumerated using a Spiral Systems Laser Counter (Model 500A, Spiral System Instruments, Inc.).

RESULTS

After an increase of less than 1 log cycle, the growth of *L. monocytogenes* Scott A was effectively suppressed by the presence of *C. piscicola* LK5 in UHT milk held at 5C (Fig. 1). The suppression of pathogen growth occurred at a time corresponding to the attainment of early stationary growth by LK5, with no bactericidal activity observed. LK5 was not active against Scott A when the incubation temperature was elevated to 19C. The response in UHT milk was similar to that observed in microbiological media (Buchanan and Klawitter 1991b).

Canned "all beef" dog food was used as a convenient model of a cooked meat product. The growth of Scott A was strongly delayed when co-cultured with LK5 at 5C (Fig. 2), with less than 1 log cycle increase in the population density of the pathogen after 400 h of incubation compared to greater than 4 log cycle increase in the *L. monocytogenes* monoculture control. Unlike the UHT milk, some activity was retained when the temperature was elevated to 19C, with approximately a 1.5 log cycle differential between the co-culture and the control.

In raw ground beef that had been sterilized by irradiation, Scott A grew readily at 19C, achieving levels of approximately 10⁸ cfu/g within 4 days (Fig. 3). When LK5 was incorporated into the temperature abused product, growth of the pathogen was severely limited and declined somewhat with extended storage. At 5C, the control culture of *L. monocytogenes* did not grow, remaining unchanged over the course of 600 h of incubation. In the co-culture, the level of Scott A fell below the lower limit of detection after approximately 300 h. It was this inactivation of *L. monocytogenes* in a refrigerated raw ground beef sample that had led originally to the isolation of LK5 (Buchanan and Klawitter 1991a).

When nonsterile ground beef with a background microflora between 10⁶-10⁷ cfu/g was evaluated, *L. monocytogenes* again did not grow at refrigera-

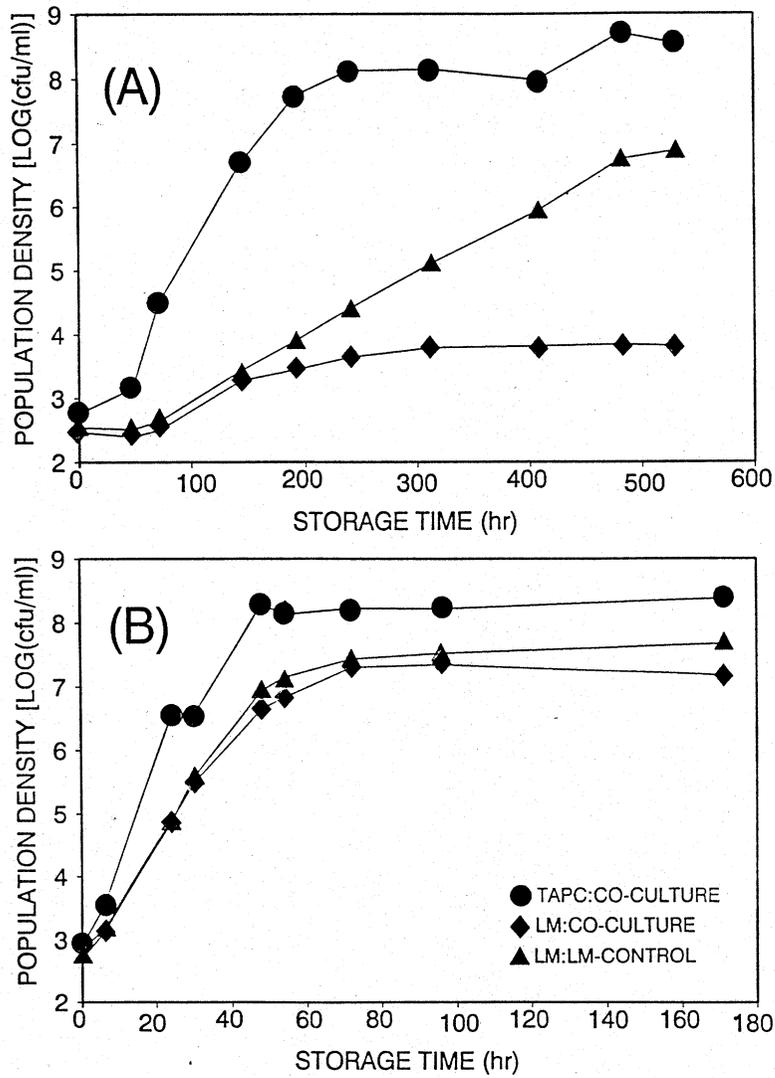


FIG. 1. EFFECT OF *CARNOBACTERIUM PISCICOLA* LK5 ON THE GROWTH OF *LISTERIA MONOCYTOGENES* SCOTT A AT 5 (A) AND 19C (B) IN UHT MILK
TAPC = total aerobic plate counts, LM = *L. monocytogenes*. Values represent the means of three replicate cultures assayed in duplicate.

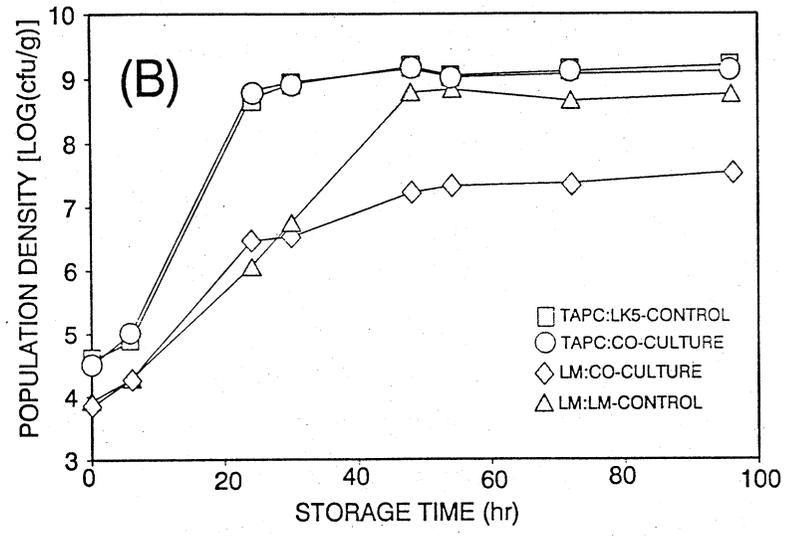
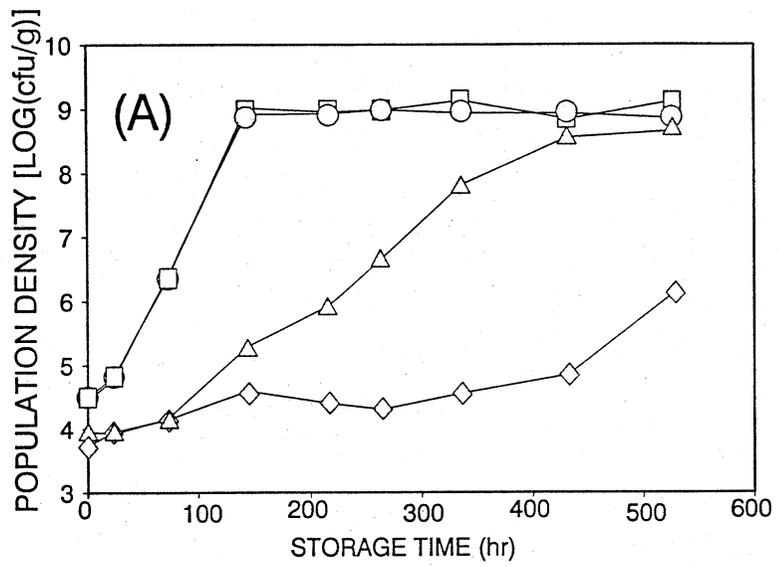


FIG. 2. EFFECT OF *CARNOBACTERIUM PISCICOLA* LK5 ON THE GROWTH OF *LISTERIA MONOCYTOGENES* SCOTT A AT 5 (A) AND 19C (B) IN CANNED, "ALL BEEF" DOG FOOD
 Values represent the means of three replicate cultures assayed in duplicate.

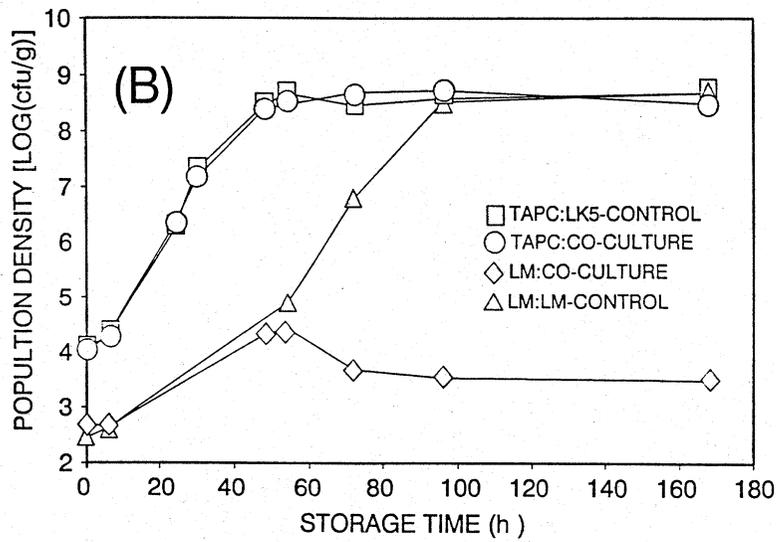
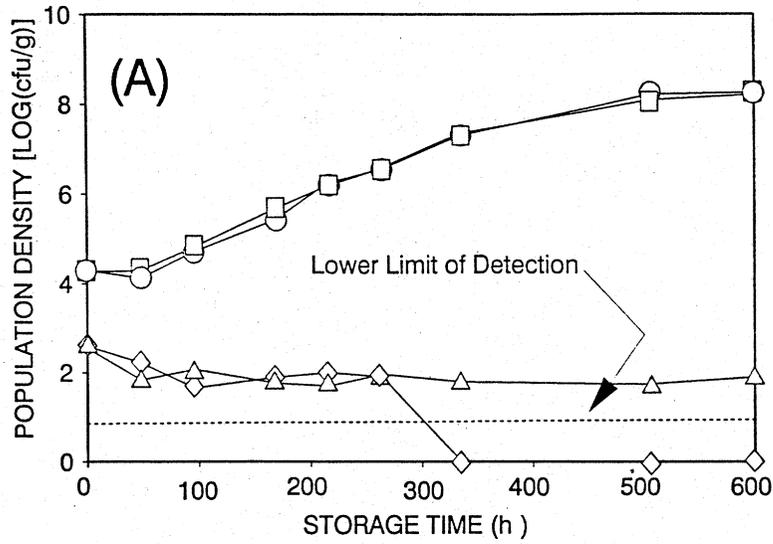


FIG. 3. EFFECT OF *CARNOBACTERIUM PISCICOLA* LK5 ON THE GROWTH OF *LISTERIA MONOCYTOGENES* SCOTT A AT 5 (A) AND 19C (B) IN RAW GROUND BEEF THAT HAD BEEN STERILIZED BY GAMMA IRRADIATION

Values represent the means of three replicate cultures assayed in duplicate.

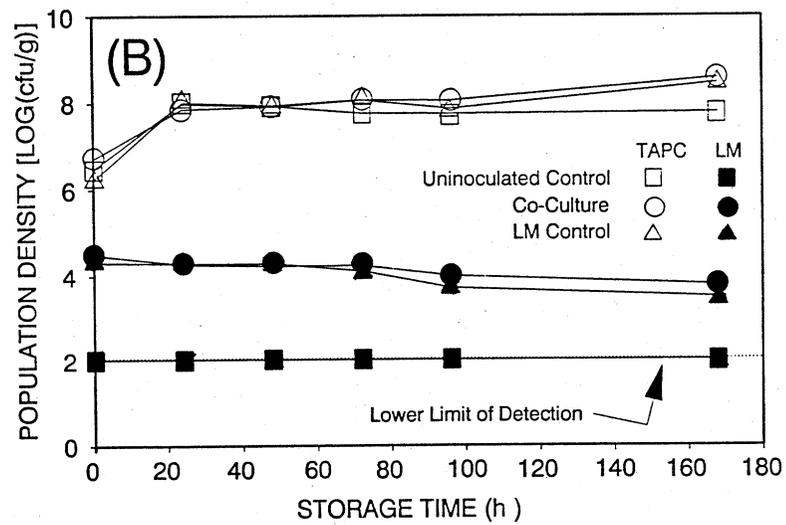
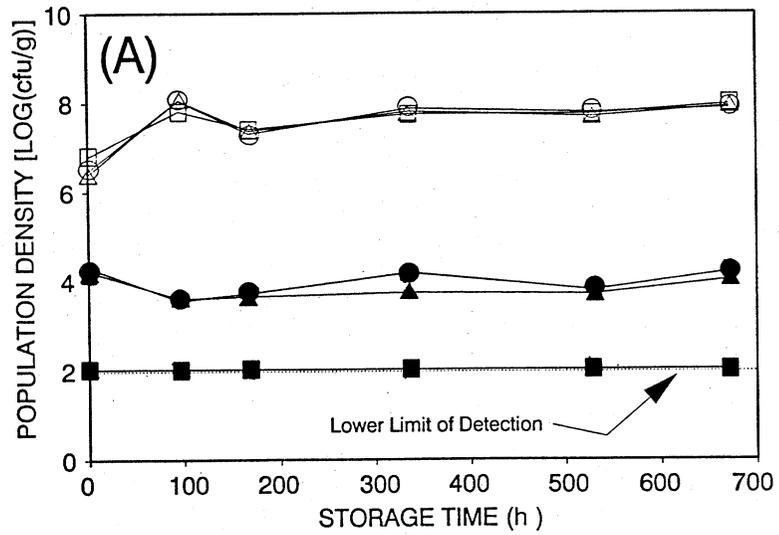


FIG. 4. EFFECT OF *CARNOBACTERIUM PISCICOLA* LK5 ON THE GROWTH OF *LISTERIA MONOCYTOGENES* SCOTT A AT 5 (A) AND 19C (B) IN RAW GROUND BEEF
 Values represent the means of three replicate cultures assayed in duplicate.

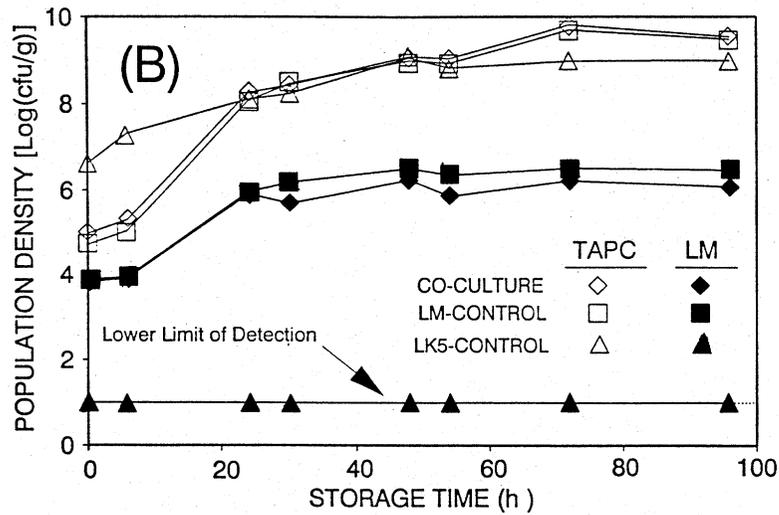
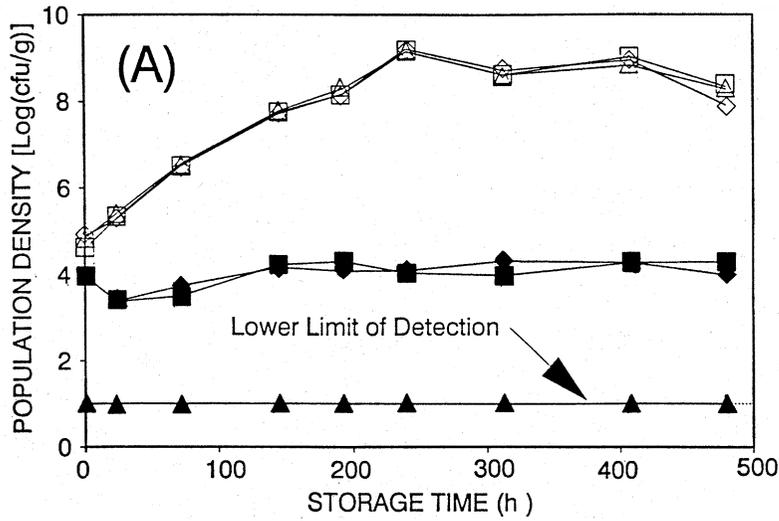


FIG. 5. EFFECT OF *CARNOBACTERIUM PISCICOLA* LK5 ON THE GROWTH OF *LISTERIA MONOCYTOGENES* SCOTT A AT 5 (A) AND 19C (B) IN SLICED CHICKEN ROLL. Values represent the means of three replicate cultures assayed in duplicate.

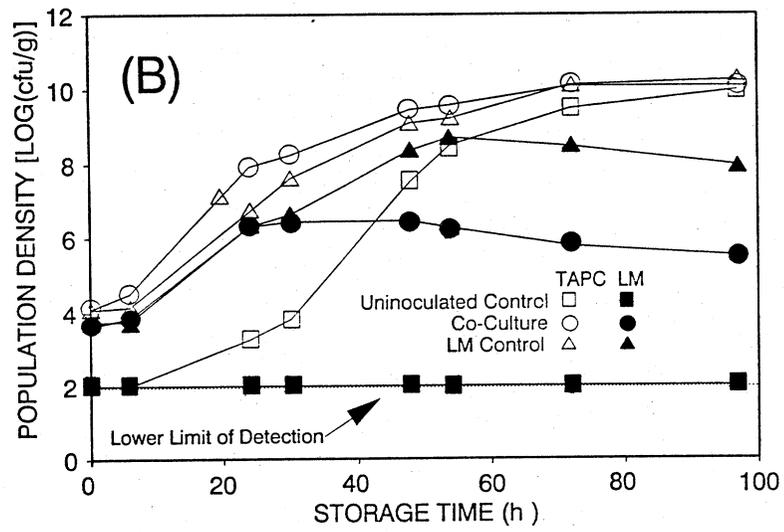
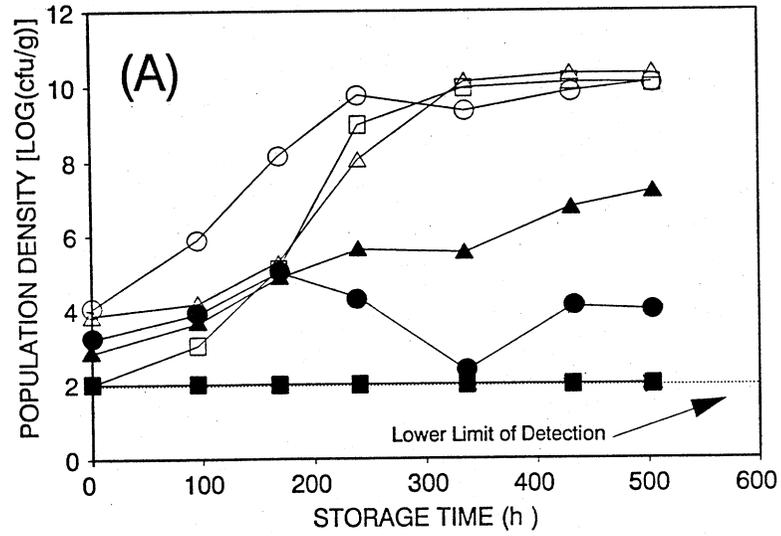


FIG. 6. EFFECT OF *CARNOBACTERIUM PISCICOLA* LK5 ON THE GROWTH OF *LISTERIA MONOCYTOGENES* SCOTT A AT 5 (A) AND 19C (B) IN PASTEURIZED CRABMEAT
 Values represent the means of three replicate cultures assayed in duplicate.

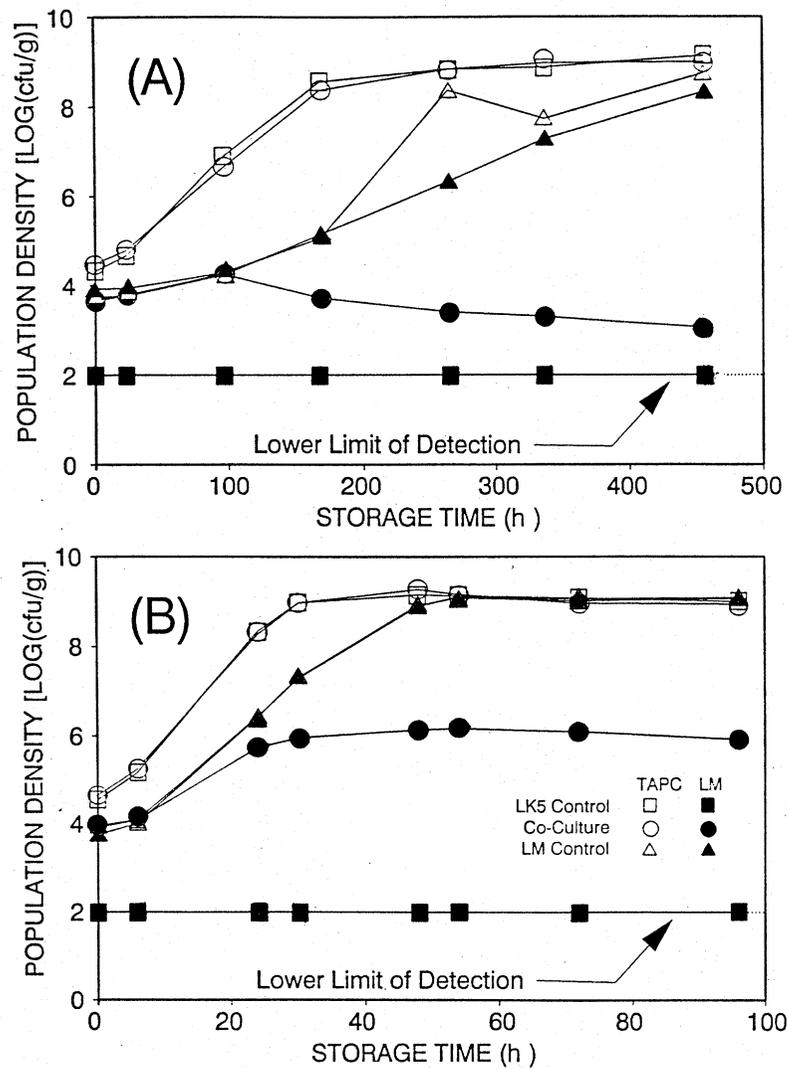


FIG. 7. EFFECT OF *CARNOBACTERIUM PISCICOLA* LK5 ON THE GROWTH OF *LISTERIA MONOCYTOGENES* SCOTT A AT 5 (A) AND 19C (B) IN CANNED CREAMED CORN
 Values represent the means of three replicate cultures assayed in duplicate.

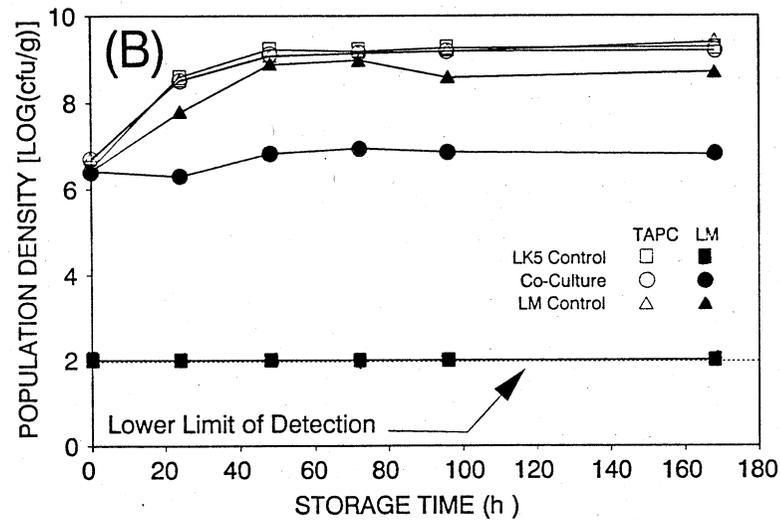
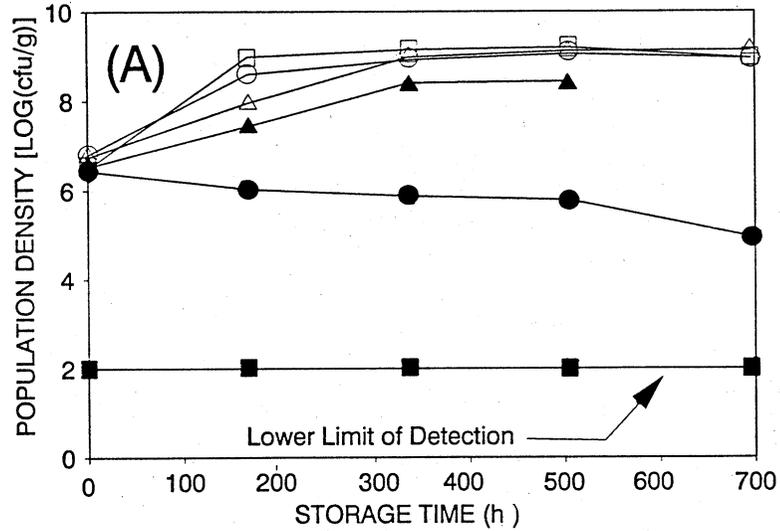


FIG. 8. EFFECT OF *CARNOBACTERIUM PISCICOLA* LK5 ON THE GROWTH OF *LISTERIA MONOCYTOGENES* SCOTT A AT 5 (A) AND 19C (B) ON THE SURFACE OF FRANKFURTERS
 Values represent the means of three replicate cultures assayed in duplicate.

tion temperatures (Fig. 4). However, unlike the sterile product, the addition of LK5 to nonsterile raw ground beef did not result in an inactivation of Scott A after extended storage, with the level of the pathogen remaining essentially unchanged over the extended incubation period. Interestingly, the pathogen also did not grow in the ground beef samples held at 19C, remaining constant over the course of a week of temperature abuse. Unlike earlier work with nonsterile ground beef (Buchanan and Klawitter 1991a), LK5 had no effect on behavior of Scott A.

Only limited growth of *L. monocytogenes* was observed in chicken roll (Fig. 5), with less than a 2 log cycle increase to 10^6 cfu/g in 19C control samples and no growth in the 5C cultures. The incorporation of LK5 did not affect the growth or survival of Scott A in this product.

The pattern of responses observed with pasteurized crabmeat (Fig. 6) and canned creamed corn (Fig. 7) was similar. *L. monocytogenes* grew at 5C in these products, reaching levels of approximately 10^7 – 10^8 cfu/g. The pathogen grew in the co-cultures only during the initial phases of the incubation; its growth being subsequently suppressed by LK5. LK5 retained substantial activity in these products when the incubation temperature was increased to 19C, with a 2–3 log cycle differential in the level of *L. monocytogenes* being observed between the co-cultures and the controls. The time when an active suppression of *L. monocytogenes* in the co-culture was observed again corresponded to the time when LK5 was initially reaching its maximal growth.

Unlike the other foods where LK5 and/or Scott A were dispersed throughout the samples, the ability of LK5 to inhibit the pathogen was examined in frankfurters using surface inoculation (Fig. 8). The efficiency of inoculation of *L. monocytogenes* and *C. piscicola* to frankfurters that had been momentarily boiled prior to inoculation was much greater than anticipated, with the level of Scott A being greater than 10^6 cfu/g. However, this high inoculum level did not adversely affect the ability of LK5 to suppress the growth of the pathogen. Though *L. monocytogenes* controls reached levels greater than 10^8 cfu/g, the pathogen did not grow on the surface of the frankfurters at either incubation temperature when cultured in the presence of LK5.

DISCUSSION

The inoculum levels employed in the current study were selected to provide a reasonably rigorous challenge to the antilisterial activity of *C. piscicola* LK5 while simultaneously employing realistic levels of *L. monocytogenes*. It is readily apparent that LK5 can effectively suppress the growth of *L. monocytogenes* in a number of food systems. There were substantial differences among the food types evaluated, with LK5 generally being more effective in foods

where the background microflora had been reduced through the use of thermal processing or irradiation. A majority of foods examined received some form of lethal treatment to both simplify interpretation of observed effects and reflect events that could occur as a result of past processing contamination by *L. monocytogenes*.

The organism was consistently more effective at the lower incubation temperature, which is similar to the results observed with microbiological media (Buchanan and Klawitter 1991b). However, a substantial degree of activity was observed at the higher incubation temperature with cooked meat (dog food), sterile raw ground beef, crabmeat, creamed corn, and frankfurters suggesting that there are factors in food that enhance the effectiveness of the antilisterial agent compared to microbiological media. One possibility that is consistent with the current results is that the solid matrix provided by a number of the foods allowed the localized accumulation of the bacteriocin.

While the bacteriocin produced by LK5 is bactericidal when applied at high concentrations (Buchanan and Klawitter 1991b), the response observed both in microbiological media and most foods was a suppression of growth. This is similar to the observations of Schillinger *et al.* (1991) who reported that a bacteriocin-producing strain of *Lactobacillus sake* gave a bactericidal response in MRS broth but yielded a bacteriostatic response in both cooked minced meat and comminuted raw cured pork. Berry *et al.* (1991) reported that the growth at 4C of a five strain mixture of *L. monocytogenes* on frankfurters was suppressed, but the pathogen was not inactivated, when the surface of the product was co-inoculated with a high level of a bacteriocin-producing strain of *Pediococcus acidilactici*. Lower inoculum levels resulted in delayed growth of the pathogen.

An exception to the pattern of bacteriostasis observed in the current study was the inactivation of Scott A in sterile raw ground beef. This is similar to the response initially observed in raw ground beef that led to the isolation of LK5 (Buchanan and Klawitter 1991a). It should be noted that the selective media employed do not detect injured cells. Additional research is needed to determine what portion of the observed inactivation in raw ground beef was actually the result of sublethal stress. The reason for the enhanced effectiveness in this product is not known, but may reflect a combination of this product being the niche where *C. piscicola* thrives and *L. monocytogenes* being stressed in this product. The lack of growth of *L. monocytogenes* in raw ground beef stored at 5C has been noted by several investigators (Buchanan *et al.* 1987; Johnson *et al.* 1988a,b; Glass and Doyle 1989; Shelef 1989; Kaya and Schmidt 1989; Buchanan and Klawitter 1991a; Benedict and Schultz 1991), even though the organism grows readily at refrigeration temperatures in cooked meat products (Fig. 2 and 8). The possibility that there is a connection between the lack of

growth of *L. monocytogenes* in refrigerated ground beef and the enhanced activity of LK5 will require additional research.

The lack of LK5 activity in the 5C/nonsterile raw ground beef, as well as the lack of growth of *L. monocytogenes* in the 19C controls were surprising (Fig. 4). The reason for this unexpected result is not known, but one possible explanation is that the microflora of the ground beef sample contained an additional bacteriocin-producing strain(s) with activity against the pathogen. This could also account for the lack of activity with LK5. Ahn and Stiles (1990) and Schillinger and Holzapfel (1990) reported that bacteriocins from one *Carnobacterium* isolate can inhibit other members of the genus, and Schillinger and Lucke (1991) reported that the bacteriocin produced by *L. sake* Lb706 inhibited *C. piscicola*. The presence of an active microflora may also account for the lack of growth of Scott A in the refrigerated chicken roll and the pathogen's relatively poor growth 19C in that product (Fig. 5). An alternative possibility is that this product contained an ingredient(s) that restricted the growth of *L. monocytogenes*.

The current study has demonstrated that *C. piscicola* LK5 can be used to control the growth of susceptible strains of *L. monocytogenes* in a variety of foods, particularly at refrigeration temperatures. The feasibility of directly employing the organism in food systems will be dependent on its organoleptic impact. Preliminary assessments have indicated that LK5 is not a strong acid producer, and did not depress the pH of foods unless there was a significant level of available carbohydrate (e.g., creamed corn). Further, LK5 did not impart a strong odor even when cultured to high levels in a bland product such as crabmeat. However, a more detailed evaluation of its impact on the quality attributes of various foods will be required. Other possible methods for taking advantage of the antilisterial activity of LK5 include directly adding isolated bacteriocin to food products or transferring the gene(s) encoding this antilisterial agent to other bacteria used for food production. Work on the isolation of the bacteriocin and the characterization of its genetics is currently underway in our laboratory.

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