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Behavior of *Listeria monocytogenes* in the Presence of Flavobacteria in Skim Milk at 7 or 13°C

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ABSTRACT

Sterile samples of skim milk were inoculated with *Listeria monocytogenes* (strain Scott A, California, or V7), *Flavobacterium lutescens* or *Flavobacterium* species, or a combination of *L. monocytogenes* plus flavobacteria and incubated at 7 or 13°C for 8 weeks. McBride Listeria agar was used to determine populations of *L. monocytogenes* at 0, 7, 14, 28, 42, or 56 d, and plate count agar was used to enumerate flavobacteria at the same time intervals. Growth of *L. monocytogenes* was significantly ($P<0.05$) enhanced during incubation at 7 or 13°C in mixed cultures with *F. lutescens*. When tested with *Flavobacterium* sp. ATCC 21429, numbers of *L. monocytogenes* decreased insignificantly ($P<0.05$) in mixed cultures compared to pure cultures. *L. monocytogenes* did not affect growth of flavobacteria; also, no marked changes in pH of the milk were caused either by *L. monocytogenes* alone or by mixed cultures.

Psychrotrophic microorganisms are important to the dairy industry because they can multiply at low temperatures and cause spoilage of milk and some milk products. *Flavobacterium* is a genus of gram-negative psychrotrophic bacteria often isolated from raw and pasteurized milk and sometimes from butter (*2*). Defects such as surface taint and apple odor in butter are attributed to flavobacteria (*4,14*). Presence of flavobacteria in mastitic udders has suggested the possible pathogenicity of certain strains (*12*), whereas their ability to detoxify aflatoxin suggests a more useful role for certain species of the genus *Flavobacterium* (*1*).

Listeria monocytogenes is a gram-positive, rod-shaped, psychrotrophic, facultative aerobe which causes listeriosis in humans and animals (*7*). Recently, several outbreaks have confirmed that human listeriosis can be indirectly transmitted from infected animals to humans through consumption of contaminated food products (*6,8,9,13*). Contamination of milk and milk products with *L. monocytogenes* and other psychrotrophic bacteria such as *Flavobacterium* is most likely to occur during handling and processing of the foods or by postprocessing contamination. Our study was undertaken to determine if presence of *Flavobacterium* sp. enhances or suppresses growth of *L. monocytogenes* in milk. These experiments also establish the influence of *L. monocytogenes* on growth of *Flavobacterium* at low temperatures as used for storage of milk and milk products.

MATERIALS AND METHODS

Preparation of cultures and samples

Three strains of *L. monocytogenes* were used in these experiments. Included were Scott A (clinical isolate, serotype 4b), V7 (milk isolate, serotype 1) both provided by R.M. Twedt, Food and Drug Administration, Cincinnati, OH, and California (serotype 4b isolate from Mexican-style cheese) obtained from Silliker Laboratories, Inc., Carson, CA. Stock cultures were maintained through bimonthly transfer on tryptose agar (Difco Laboratories, Detroit, MI) and storage at 4°C.

To prepare for an experiment, inocula from stock cultures were transferred to tryptose broth (TB) (Difco), and incubated aerobically for 24 h at 35°C. These cultures were transferred to new TB which was incubated as just described. Inocula (0.1 ml) from these TB cultures were added to 500-ml screw-capped Erlenmeyer flasks, each containing 200 ml of autoclaved (15 min, 121°C) skim milk. Following incubation for 24 h at 35°C, 0.1 ml of the milk culture was transferred into each of two 500-ml Erlenmeyer flasks containing 200 ml of sterile skim milk. Incubation of flasks and their contents was for 48 h at 35°C. One of these cultures was diluted to ca. 10^5 *L. monocytogenes*/ ml and then was used as starting inoculum for test substrates.

Preparation of flavobacteria cultures

Two strains of flavobacteria were used in these experiments. Included were *Flavobacterium lutescens* ATCC 27951 which was initially isolated from yogurt and *Flavobacterium* sp. ATCC 21429 which produces ampicillin by conversion. The flavobacteria were obtained from the American Type Culture Collection (Rockville, MD) as freeze-dried cultures. Stock cultures were maintained on slants of nutrient agar (Difco), stored at 4°C, and were transferred bimonthly.

Preparation of flavobacteria cultures and inoculation of milk samples were done as just described for *L. monocytogenes* cultures except the incubation temperature for *F. lutescens* was 30°C and for *Flavobacterium* sp. it was 26°C. The following pure or mixed cultures of these microorganisms, each in 200 ml of sterile skim milk, were prepared: (a) *L. monocytogenes* strain Scott A, (b) *L. monocytogenes* strain California (CA), (c) *L. monocytogenes* strain V7, (d) *F. lutescens*, (e) *Flavobacterium* sp., (f) *L. monocytogenes* (Scott A) + *F. lutescens*, (g) *L. monocytogenes* (CA) + *F. lutescens*, (h) *L. monocytogenes* (V7) + *F. lutescens*, (i) *L. monocytogenes* (Scott A) + *Flavobacterium* sp., (j) *L. monocy-*

togenes (CA) + *Flavobacterium* sp., and (k) *L. monocytogenes* (V7) + *Flavobacterium* sp. Mixed cultures were prepared to contain approximately equal numbers of the two organisms. Cultures were held at 7 or 13°C for 56 d.

Enumeration of L. monocytogenes *and* Flavobacterium *species*

Flasks were agitated just before sampling; 1-ml portions from each well-mixed culture were appropriately diluted in sterile 0.5% peptone (Difco) solution, followed by duplicate surface plating of 0.1 ml of specific dilutions on McBride Listeria agar (*11*). Plate count agar (PCA) (Difco) was used to enumerate flavobacteria. It was easy to distinguish between colonies of flavobacteria and listeriae on PCA, both by color and size. The *Flavobacterium* colonies were larger than *Listeria* colonies and were yellow-cream rather than blue as is typical for *L. monocytogenes*. Samples also were taken at the beginning and end of experiments and pH was determined using a pH meter (Corning Model 10).

Statistical analysis

Data from 0, 7, 14, 28, 42, and 56 d were analyzed through use of the Statistical Analysis System (SAS) computer program (SAS Institute Inc. SAS Circle, Box 8000, Cary, NC 27512).

RESULTS AND DISCUSSION

Growth of *L. monocytogenes* strains Scott A, CA, and V7 was enhanced in milk by the presence of *F. lutescens* during storage at 7°C for 56 d (Fig. 1A); this enhancement was significant ($P < 0.05$) during the incubation period from 14-42 d. These results are similar to those observed by Marshall and Schmidt (*10*) when they studied growth of *L. monocytogenes* at 10°C in milk preincubated with selected pseudomonads. The results also are similar to our earlier data obtained when *L. monocytogenes* grew in the presence of *Pseudomonas fluorescens* at 7 or 13°C in skim milk (*5*). However, growth of *L. monocytogenes* was enhanced by flavobacteria throughout the storage period, whereas such enhancement was observed only after 7 and 14 d when *P. fluorescens* grew together with *L. monocytogenes*.

The maximum population (Table 1) (as indicated by orders of magnitude) of *L. monocytogenes* strains Scott A, CA, and V7 in the presence of *F. lutescens* during storage at 7°C was 3.7, 3.6, and 3.3, respectively, compared to 3.3, 3.3, and 3.0 in the absence of *F. lutescens*. Proteolytic activity of flavobacteria in milk is generally thought to be responsible for providing substances that enhance growth or activity of other organism (*3*). *F. lutescens* grew well in skim milk at 7°C throughout the storage period (56 d) with or without *L. monocytogenes* (Fig. 1B).

Statistical analysis of results obtained at 13°C indicates that the behavior of *L. monocytogenes* in the presence of *F. lutescens* was similar to that at 7°C. Thus, growth of *L. monocytogenes* was significantly ($P < 0.05$) enhanced by the presence of *F. lutescens* (Fig. 2A). Also, there was no effect by *L. monocytogenes* on growth of *F. lutescens* (Fig. 2B).

Statistically, there was no significant ($P<0.05$) effect of *Flavobacterium* sp. ATCC 21429 on the three strains of *L. monocytogenes* during storage at 7°C (Fig. 3A). The maximum population of *L. monocytogenes* strain Scott A (as indicated by orders of magnitude over that of the initial number) at 7°C was 2.9, whereas in the presence of *Flavobacterium* sp. it was 3.2. Thus, there was a slight increase in population of ca. 0.3 order of magnitude in the mixed compared to the pure culture (Table 1). In contrast, maximum populations of strains CA and V7 decreased

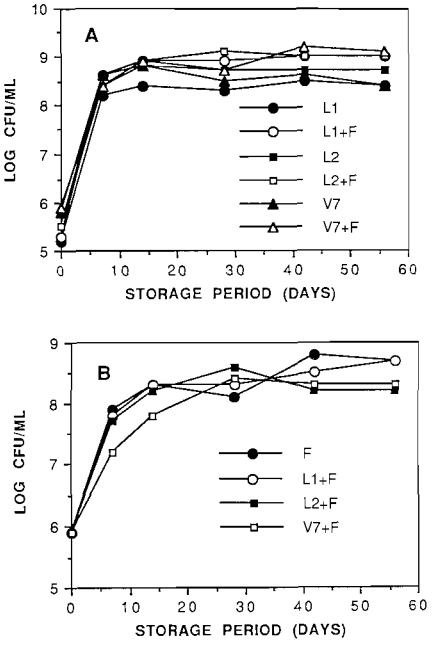

Figure 1. *Behavior of* L. monocytogenes *alone and in the presence of* F. lutescens *ATCC 27951 in skim milk held at 7°C for 56 d. L1* = L. monocytogenes *strain Scott A, L2* = L. monocytogenes *strain California, V7* = L. monocytogenes *strain V7 and F1* = F. lutescens *(A), and behavior of* F. lutescens *in the presence of* L. monocytogenes *in skim milk held at 7°C (B). See Fig. 1 (A) for explanation of symbols.*

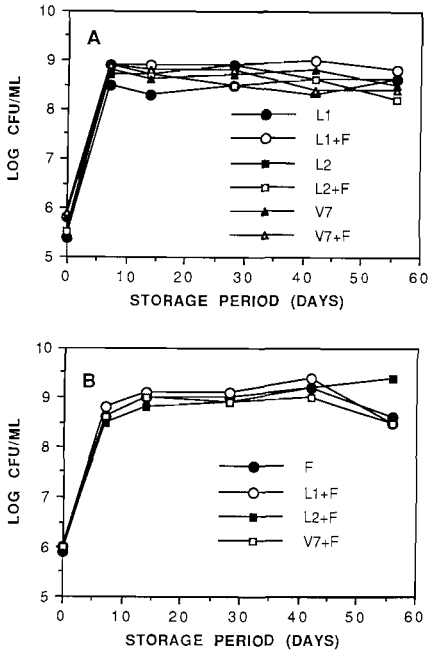

Figure 2. *Behavior of* L. monocytogenes *alone and in the presence of* F. lutescens *ATCC 27951 in skim milk held at 13°C for 56 d (A), and behavior of* F. lutescens *in the presence of* L. monocytogenes *in skim milk held at 13°C for 56 d (B). See Fig. 1 (A) for explanation of symbols.*

TABLE 1. *Changes in populations of* L. monocytogenes *(as indicated by order of magnitude increase) in the presence of* Flavobacterium *species when incubated in skim milk at 7 or 13°C*[a].

Inoculum	7°C			13°C		
	Initial number (log_{10}/ml)	Maximum number (log_{10}/ml)	Increase (orders of) magnitude)	Initial number (log_{10}/ml)	Maximum population (log_{10}/ml)	Increase (orders of magnitude)
L1[b]	5.2	8.5	3.3	5.4	8.6	3.2
L1 + F1[c]	5.3	9.0	3.7	5.8	9.0	3.2
L2[d]	5.5	8.8	3.3	5.5	8.9	3.4
L2 + F1	5.5	9.1	3.6	5.5	8.9	3.4
V7[e]	5.8	8.8	3.0	5.8	8.8	3.0
V7 + F1	5.9	9.2	3.3	5.9	8.9	3.0
L1	5.7	8.6	2.9	5.6	9.1	3.5
L1 + F2[f]	5.7	8.9	3.2	5.6	8.8	3.2
L2	5.6	9.0	3.4	5.3	8.6	3.3
L2 + F2	5.7	8.8	3.1	5.9	8.7	2.8
V7	5.9	9.1	3.2	5.4	8.8	3.4
V7 + F2	5.8	8.6	2.8	5.5	8.8	3.3

[a] = Average of three trials.
[b] L1 = *L. monocytogenes* strain Scott A.
[c] F1 = *F. lutescens* ATCC 27951.
[d] L2 = *L. monocytogenes* strain California.
[e] V7 = *L. monocytogenes* strain V7.
[f] F2 = *Flavobacterium* sp. ATCC 21429.

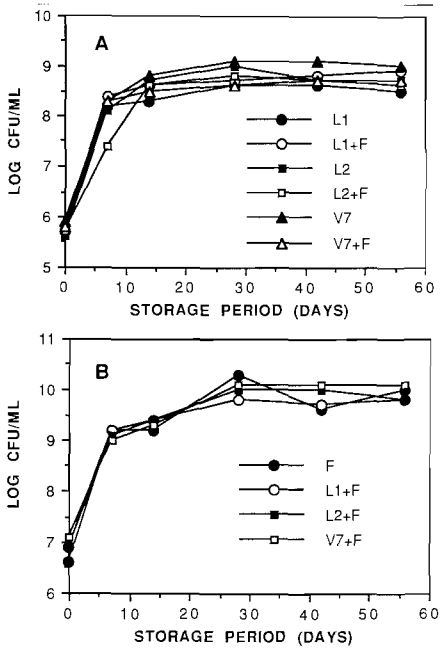

Figure 3. *Behavior of* L. monocytogenes *alone and in the presence of* Flavobacterium *sp. ATCC 21429 in skim milk held at 7°C for 56 d (A), and behavior of* Flavobacterium *sp. in the presence of* L. monocytogenes *in skim milk held at 7°C for 56 d (B). L1* = L. monocytogenes *strain Scott A, L2* = L. monocytogenes *strain California, V7* = L. monocytogenes *strain V7 and F2* = Flavobacterium *sp. ATCC 21429.*

somewhat in the presence of *Flavobacterium* sp. At 7°C, *Flavobacterium* sp. ATCC 21429 grew well in skim milk with or without *L. monocytogenes* (Fig. 3B).

At 13°C, and with the same strain of *Flavobacterium,* results (Fig.4A) indicate that populations of *L. monocytogenes* strains Scott A, CA, and V7 decreased by ca. 0.3, 0.5, and 0.1 order of magnitude, respectively, compared to controls. The decrease was more pronounced for strain CA than for the other strains tested (Table 1). Our results suggest that growth of *L. monocytogenes* was inhibited slightly by *Flavobacterium* sp. ATCC 21429. Data in Fig. 4B show that *Flavobacterium* sp. ATCC 21429 grew well in skim milk during storage at 13°C in the presence or absence of *L. monocytogenes.*

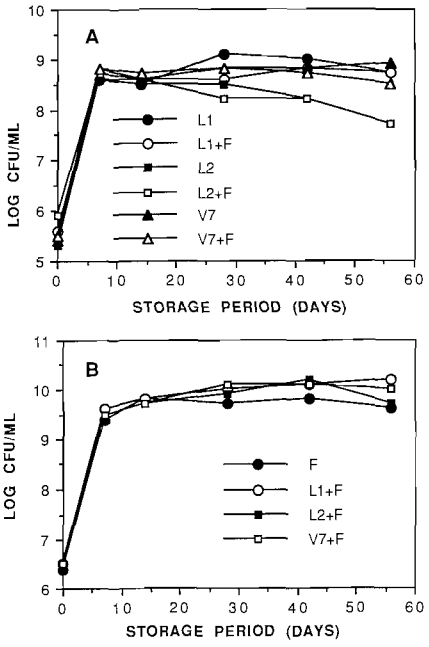

Figure 4. *Behavior of* L. monocytogenes *alone and in the presence of* Flavobacterium *sp. ATCC 21429 in skim milk held at 13°C for 56 d (A), and behavior of* Flavobacterium *sp. in the presence of* L. monocytogenes *in skim milk held at 13°C for 56 d (B). See Fig. 3, for explanation of symbols.*

Measurement of pH at the beginning and end of experiments revealed no marked changes during the incubation period either for pure or mixed cultures (data not shown). In no instance did the pH drop by more than 0.5 unit at the end of incubation at 7 or 13°C.

In conclusion, *L. monocytogenes* grew effectively in the presence of common psychrotrophic bacteria in the genus *Flavobacterium*. Some inhibition or enhancement of growth by *L. monocytogenes* was noted, depending on the strain of *Flavobacterium* being tested. Results also indicate that the two strains of *Flavobacterium* tested in this study grew well in skim milk in the presence or absence of *L. monocytogenes*.

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