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Postenrichment Population Differentials Using Buffered *Listeria* Enrichment Broth: Implications of the Presence of *Listeria innocua* on *Listeria monocytogenes* in Food Test Samples†

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Abstract

The recovery of low levels of *Listeria monocytogenes* from foods is complicated by the presence of competing microorganisms. Nonpathogenic species of *Listeria* pose a particular problem because variation in growth rate during the enrichment step can produce more colonies of these nontarget cells on selective and/or differential media, resulting in a preferential recovery of nonpathogens, especially *Listeria innocua*. To gauge the extent of this statistical barrier to pathogen recovery, 10 isolates each of *L. monocytogenes* and *L. innocua* were propagated together from approximately equal initial levels using the current U.S. Food and Drug Administration's enrichment procedure. In the 100 isolate pairs, an average 1.3-log decrease was found in the 48-h enrichment *L. monocytogenes* population when *L. innocua* was present. In 98 of the 100 isolate pairs, *L. innocua* reached higher levels at 48 h than did *L. monocytogenes*, with a difference of 0.2 to 2.4 log CFU/ml. The significance of these population differences was apparent by an increase in the difficulty of isolating *L. monocytogenes* by the streak plating method. *L. monocytogenes* went completely undetected in 18 of 30 enrichment cultures even after colony isolation was attempted on Oxoid chromogenic *Listeria* agar. This finding suggests that although both *Listeria* species were present on the plate, the population differential between them restricted *L. monocytogenes* to areas of the plate with confluent growth and that isolated individual colonies were only *L. innocua*.

Despite the relatively low number of annual incidences of foodborne illness caused by *Listeria monocytogenes*, this organism remains a human health concern because of its ability to grow in many different foods stored at low temperatures and the severity of the illness that it can cause, particularly in susceptible populations. Although the use of molecular-based detection platforms is increasing in the area of food safety, traditional culture-based methods are still more commonly used for the analysis of official test samples, including those

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suspected of contamination with *L. monocytogenes*. To detect and recover low levels of foodborne pathogens, a 1- to 2-day enrichment period is normally used. The ideal selective enrichment medium establishes a growth environment in which the target organism is capable of multiplying while populations of nontarget organisms remain stagnant or decline. Unfortunately, selective enrichment media rarely, if ever, have absolute specificity. Thus, target and uninhibited nontarget organisms compete for growth space, which can ultimately affect recovery of foodborne pathogens particularly when highly similar species such as *L. monocytogenes* and *Listeria innocua* are both present in the test sample.

In an earlier published work *(16)*, the presence of *L. innocua* hindered the recovery of *L. monocytogenes* during growth in selective enrichment broth formulated for recovery of total *Listeria* species. In UVM broth inoculated with similar levels of both species, *L. innocua* populations were approximately 1.1-log higher than those of *L. monocytogenes*. In Fraser broth, final *L. innocua* populations were 1.24- to 2.57-log higher than those of *L. monocytogenes*. Similar observations were made when these two strains were coinoculated into cheese sauce and enumerated at 24 and 48 h during a two-step enrichment process.

Additional studies indicated a diminished ability to detect or recover *L. monocytogenes* from food products that had also been artificially contaminated with *L. innocua (7)*. When *L. innocua* was present, *L. monocytogenes* was recovered from only 45 of 98 inoculated frozen beef broth samples. In the absence of *L. innocua*, the recovery of *L. monocytogenes* was 100%. The growth of *L. monocytogenes* and *L. innocua* during selective enrichment of cultures from naturally contaminated foods also has been investigated *(2)*. No obvious correlations were observed between growth rate and final enrichment populations of each *Listeria* species or between the ability to produce inhibitory substances and the populations of each *Listeria* species during the enrichment period. A similar situation also was reported for artificially contaminated milk *(6)*. The presence of *L. innocua* in test samples can hinder both the detection and recovery of *L. monocytogenes*. Zitz et al. *(18)* reported a significant decrease in the detection of low levels of *L. monocytogenes* in mixed cultures with *L. innocua* at an initial ratio of 2:1 when using a commercial automated immunofluorescence detection system.

Efficient recovery of *L. monocytogenes* from test samples that are also contaminated with *L. innocua* remains an unresolved issue particularly for regulatory agencies for whom recovery of the organism is preferred over simple detection of biomarkers that are only suggestive of the presence of viable or nonviable organisms. Differences in growth rate resulting from species variation in resistance to selective agents such as acriflavin and the production of inhibitory substances by competing organisms likely affect the outcome of selective enrichment cultures. However, the current literature indicates that the resistance to selective agents and production of inhibitory substances are not sufficient to account for the differences in *Listeria* species recovery. Additional studies using a larger number of isolates and various food matrices are necessary to fully understand how the presence of nonpathogenic *Listeria* species affects the recovery of *L. monocytogenes*.

The aim of the present study was to monitor the growth and recovery of *L. monocytogenes* serogroup 4b strains and *L. innocua* in buffered *Listeria* enrichment broth during a 48-h

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enrichment period, which is a commonly used procedure for recovery of *L. monocytogenes*. Differential enumeration and colony isolation by the streak plating method on various selective and differential media were used to determine the extent of preferential recovery of one *Listeria* species over the other. The contribution of individual isolate growth rates and the ability of *L. innocua* to produce inhibitory substances was determined. A streptomycin-resistant strain of *L. monocytogenes* Scott A was used to determine the effects of preenrichment levels of each *Listeria* species on the final 48-h enrichment population and on the ability to recover the pathogen by the streak plating method.

Although competition among *Listeria* species during selective enrichment has been reported, the present study was focused on the U.S. Food and Drug Administration (FDA) method and determining the actual population differentials between the competitor and target microorganisms, which relates to both recovery and detection. This work extends earlier observations by including a much larger set of *L. monocytogenes* and *L. innocua* isolate pairs, all originating from food or food processing environments. The population differentials provide a more quantitative assessment of the failure to recover *L. monocytogenes* in the presence of *L. innocua* than simple positive and negative recovery results. The large population differentials between these two species indicate that the use of chromogenic media may not solve the problem of *L. monocytogenes* recovery.

MATERIALS AND METHODS

Listeria isolates

All isolates used in this study were obtained from food products or food processing environments using conventional culture, isolation, and confirmation procedures described in the FDA *Bacteriological Analytical Manual* (BAM) *(12)*. In addition to traditional biochemical testing, the API *Listeria* kit (bioMérieux, Inc., Hazelwood, MO), the Vitek II bacteria identification system (bioMérieux), and conventional PCR of species-specific gene targets were used for identification. All isolates were stored long term in brain heart infusion broth supplemented with 30% glycerol at −80°C. Daily working stocks were maintained at room temperature in motility agar tubes. New working stock cultures were generated every 6 months from the freezer stocks. All *Listeria* isolates were numbered consecutively in the order in which they were recovered from food or food processing environmental samples. Gaps in the numerical sequences for *L. monocytogenes* are the result of the inclusion of only serogroup 4 isolates in this study and reflect the recovery of a different serotype.

Individual growth and generation times of experimental isolates

To estimate both exponential growth generation times and 48-h enrichment populations, an 18- to 24-h culture was established in Trypticase soy broth supplemented with yeast extract (TSBYE) *(12)* and incubated at 35°C. Serial dilutions were performed in Butterfield's phosphate buffer *(12)*, and 100 μl from the 10^{-6} dilution was used to inoculate 24.9 ml of antibiotic-supplemented buffered *Listeria* enrichment broth (BLEB) *(12)*. Unless otherwise stated, the BLEB used throughout this study was supplemented with acriflavin HCl (10 mg/liter), sodium nalidixate (40 mg/liter), and cycloheximide (50 mg/ml) at 0 h of incubation. Additional aliquots (100 μl) of the 10^{-6} dilution of the original 24-h culture also were

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surface plated in triplicate onto Trypticase soy agar supplemented with yeast extract (TSAYE) *(12)* and incubated at 35° for 24 h to establish the inoculum level that had been added to the BLEB enrichment. All enrichments were incubated for 48 h at 30°C, and the final population was determined by surface plating serial dilutions onto TSAYE. A two-sample *t* test was used to determine whether there was a significant difference in 48-h population means between *L. monocytogenes* and *L. innocua*. To determine the generation times during exponential growth, 2.5 ml of inoculated BLEB was added in triplicate to individual wells of a 24-well microplate. Growth at 30°C was monitored by turbidity (λ = 600 nm) every 0.5 h for 48 h using a Synergy HT microplate reader (BioTek, Inc., Winooski, VT). The optical density values were converted to log CFU per milliliter using a standard curve of known cell levels and their corresponding optical density values. The growth rate was determined based on the change in cell levels at optical density values of 0.2 and 0.4 and were analyzed by the following equation: $\mu = 2.303(N_{0.4} - N_{0.2})/(t_2 - t_1)$, where N is the cell population at an optical density of 0.2 or 0.4 (log CFU/ml) and t is the time (hours). Generation times (GT) were obtained by the following equation: $GT = \ln(2)/\mu$. All analyses were performed using SigmaPlot (Systat Software, Inc., San Jose, CA). A two-sample *t* test was used to determine whether there was a significant difference in the mean generation time between *L. monocytogenes* and *L. innocua*.

Listeria serogroup determination

The serotype or serogroup for all *L. monocytogenes* isolates used in this study was determined by the method of Doumith et al. *(5, 9, 10)*, which involves slide agglutination with type 1 and type 4 antisera and multiplex PCR. One limitation of the PCR-based method is that the 4b serogroup isolates (4b, 4d, and 4e serotypes) cannot be further subdivided into their actual serotypes. The primer sequences and the agarose gel banding patterns associated with each of the four major food-associated serotypes (1/2a, 1/2b, 1/2c, and 4b) are thoroughly described elsewhere *(5, 9)*. Because all isolates had been previously identified to species, the genus-specific primer set targeting the *prs* gene described in the original publication *(9)* was omitted from this study, resulting in a fourplex PCR assay. Slide agglutination using type 1 or type 4 antisera was performed, as per the manufacturer's instructions, for final serotype determination within each PCR serogroup. *L. monocytogenes* isolates in which the serotypes had been determined previously using traditional slide agglutination serotyping (Denka Seiken USA, Inc., Campbell, CA) were used as amplification controls. The multiplex PCR used in this study is specific to *L. monocytogenes*; therefore, the serotypes of the *L. innocua* isolates could not be determined by this method.

Listeria mixed-species enumeration

A 100-μl aliquot from the 10^{-6} dilution of both *L. monocytogenes* and *L. innocua* prepared as described above was added to a 50-ml polystyrene conical tube containing 24.8 ml of antibiotic-supplemented BLEB. The final inoculum levels were determined ex post facto by plate count. A total of 100 *Listeria* isolate pairs were created in triplicate. The BLEB tubes were statically incubated for 48 h at 30°C. Final species population differentials were determined by surface plating onto a TSA plate containing 5% sheep blood (BAP; Remel Products, Thermo Fisher Scientific, Lenexa, KS). Both hemolytic (presumptive *L.*

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monocytogenes) and nonhemolytic (presumptive *L. innocua*) colonies were individually enumerated. Species identification was confirmed using a multiplex PCR assay *(3, 4)* on all hemolytic colonies and 10 nonhemolytic colonies per countable plate. A two-sample *t* test was used to determine whether there was a significant difference between the mean populations for isolate pairs that included *L. innocua* strains that either did or did not have produce inhibitory substances against *L. monocytogenes*.

Inhibitory activity of *L. innocua* isolates

Three previously published methods *(1, 14, 17)* were used to screen all 10 *L. innocua* isolates for the ability to produce inhibitory substances against *L. monocytogenes*. For the first method, *L. monocytogenes* was surface plated onto TSAYE plates, which were then spot inoculated with 10 µl of *L. innocua* so that all 10 L. innocua isolates could be simultaneously tested against each *L. monocytogenes* isolate on a single plate. The plates were checked at 24 h for *L. innocua* zones of inhibition within the bacterial lawn resulting from overgrowth by *L. monocytogenes*. A second method involved filtered, sterilized culture supernatants from 48-h cultures of *L. innocua*. *L. monocytogenes* was spread onto a TSAYE plate, and then culture filtrates (10 µl) from all 10 *L. innocua* isolates were spotted onto the surface of the plate. The plates were periodically checked for reduced growth of *L. monocytogenes* at the site of each spot. In the third method, 20 µl from overnight cultures of each *L. innocua* isolate was spotted onto the surface of individual TSAYE plates and incubated for 48 h at 35°C. The colonies were then removed from the agar with sterile cotton swabs, and the surface of the plate was sterilized by exposure to chloroform vapors *(1)*. *L. monocytogenes* was then added to the chloroform-sterilized plates and incubated at 35°C for 24 h. The plates were observed for reduced *L. monocytogenes* growth at the site of the original *L. innocua* spots.

Recovery of *L. monocytogenes* by the streak plate method

L. innocua ARL-Ln-010 was used to simultaneously spike refrigerated guacamole with each of the 10 *L. monocytogenes* isolates. Enrichments were conducted as described in the BAM. Streak plates were made with TSAYE, Oxford agar (BD, Franklin Lakes, NJ), PALCAM agar (BD) and Oxoid chromogenic *Listeria* agar (Remel Products). Oxford and PALCAM agar plates were incubated for 24 h at 35°C *(12)*. Oxoid chromogenic *Listeria* agar plates were incubated following the manufacturer's recommendation of 24 h at 37°C. All plates were viewed under 4× illuminated magnification. When more than one colony type was present that conformed to the definition of a typical *Listeria* colony, an attempt was made to select equal numbers of both colony types. Ten well-isolated typical *Listeria* colonies were selected from each plate and transferred to the surface of a BAP for presumptive identification based on hemolytic activity.

PCR *Listeria* species confirmation

Species confirmation was performed by multiplex PCR assay *(3, 4)*. The PCR was modified such that only the primer sets required for identifying *L. monocytogenes* and *L. innocua* were used. Bacterial genomic DNA was prepared. Typical PCRs consisted of 0.25 µl of each primer, 1.0 µl of genomic DNA, and 23 ml of a PCR master mix prepared using OmniMix HS PCR beads (Takara Bio, Inc., Otsu, Shiga, Japan) following the manufacturer's

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guidelines. Target amplification was achieved using a Veriti 96-well thermal cycler (Applied Biosystems, Foster City, CA) with the following run parameters: initial denaturation at 95°C for 1 min, denaturation at 95°C for 30 s, annealing at 60°C for 30 s, primer extension at 72°C for 90 s, and a final extension at 72°C for 5 min.

Effect of preenrichment species levels

To determine the effects of the preenrichment levels of each species, a spontaneous streptomycin-resistant variant of *L. monocytogenes* strain Scott A (CFSAN-82; FDA, Center for Food Safety and Applied Nutrition, College Park, MD) was used, which allowed the population of *L. monocytogenes* to be determined by growth on plates containing 200 μg/ml streptomycin sulfate *(8, 13)*. *L. innocua* ARL-Ln-001 was selected to be paired with *L. monocytogenes* CFSAN-82 because of its apparent lack of inhibitory activity, which was subsequently verified by the deferred antagonism plate assay. *L. monocytogenes* CFSAN-82 and *L. innocua* ARL-Ln-001 were prepared as described above and used to inoculate 24.8 ml of antibiotic-supplemented BLEB. The actual inoculum levels were determined ex post facto by plate counts. Following incubation at 30°C for 48 h, the populations of total *Listeria* and of *L. monocytogenes* specifically were determined using Oxford agar and TSAYE supplemented with 200 μg/ml streptomycin sulfate, respectively. Oxford agar and streptomycin plates were incubated for up to 48 h at 35°C, and initial colony counts were made at 24 h. To determine how preenrichment levels of both species affected *L. monocytogenes* recovery by standard isolation methods, streak plates of the enrichment cultures were analyzed as described above.

RESULTS AND DISCUSSION

Listeria isolates

Ten isolates each of both *L. monocytogenes* and *L. innocua* were obtained from naturally contaminated food products or food processing environments (Table 1) using the procedures outlined for regulatory testing *(12)*. Isolates with the same product description are from unrelated product samples. All isolates were grown individually in BLEB with selective supplements. The initial inocula for *L. monocytogenes* were 1.7 to 2.6 log CFU/ml (mean ± SD, 2.1 ± 0.1 log CFU/ml; individual inoculum levels not shown). The 48-h populations in *L. monocytogenes* pure cultures were 8.9 to 9.2 log CFU/ml (9.1 ± 0.1 log CFU/ml) (Table 1). The generation times for *L. monocytogenes* during the period of exponential growth at 30°C in BLEB with selective supplements were 1.5 to 2.4 h (1.9 ± 0.3 h) (Table 1). These times are generally in agreement with previously published times. Others have reported generation times for *L. monocytogenes* (including some serotype 4b isolates) of 1.3 to 1.6 h in BLEB under similar incubation conditions *(11)*. MacDonald and Sutherland *(15)* reported a mean generation time of 1.2 h for six *L. monocytogenes* isolates in *Listeria* enrichment broth at 30uC. The slightly longer generation times observed in the present study can be attributed to the use of 24-well microplates for growth of the cells. The increased surface area–to–volume ratio with the 24-well plate permits more aeration, which could affect the growth of *L. monocytogenes*, which is fundamentally a microaerophilic organism. Although the plates were incubated statically during the enrichment step, it was necessary to agitate

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the plates just before making the optical density measurements to ensure a homogenous suspension of cells, further increasing aeration.

The initial inoculum levels of *L. innocua* were 1.3 to 2.4 log CFU/ml (2.2 ± 0.3 log CFU/ml; individual inoculum levels not shown). The 48-h populations in *L. innocua* pure cultures were 9.1 to 9.3 log CFU/ml (9.2 ± 0.06 log CFU/ml) (Table 1). The 48-h enrichment populations for both *L. innocua* and *L. monocytogenes* were normally distributed but did not have equal variance. The Mann-Whitney rank sum test was subsequently used to determine that the median enrichment populations of the two species was significantly different ($P = 0.002$) at 9.0 and 9.2 log CFU/ml for *L. monocytogenes* and *L. innocua*, respectively. The generation times during exponential growth for *L. innocua* were 0.9 to 1.7 h (1.2 ± 0.2 h) (Table 1). These times were in close agreement with the generation time previously reported by MacDonald and Sutherland *(15)* for *L. innocua* (0.9 h). The slightly longer generation times for *L. innocua* in the present study again can be attributed to increased aeration due to the use of 24-well microplates; this aeration could be slightly inhibitory to microaerophilic organisms. The generation times for both *L. innocua* and *L. monocytogenes* were normally distributed with equal variance. The difference in the mean generation time between these two species (0.7 h) was significant ($P \leq 0.001$).

Listeria mixed species enumeration

The initial inocula were 50 to 400 CFU/25 ml for *L. monocytogenes* and 20 to 250 CFU/25 ml for *L. innocua* as determined by plate count. The 48-h populations for *L. monocytogenes* in this mixed culture enrichment were 7.0 to 9.0 log CFU/ml (7.8 ± 0.6 log CFU/ml). Because of differences in populations between the two species at 48 h, it was frequently necessary to use plates on which there were fewer than 25 colonies of *L. monocytogenes*; therefore, the counts and all calculations using those plates are only estimates, according to standard plate counting rules. Overall, the mean 48-h population of *L. monocytogenes* was approximately 1.3-log lower when cocultured in BLEB with *L. innocua* (Table 2). The 48-h populations of *L. innocua* were 8.3 to 9.7 log CFU/ml (9.2 ± 0.2 log CFU/ml). The mean 48-h populations of *L. innocua* were similar ($P > 0.05$; two-sample *t* test) in the pure culture and mixed culture enrichments, although more variability (i.e., wider range of values) was observed when *L. monocytogenes* was present. In 98 of 100 isolate pairs, *L. innocua* reached higher levels at 48 h than did *L. monocytogenes*, with a difference of 0.2 to 2.4 log CFU/ml (1.5 ± 0.6 log CFU/ml) (Table 2).

Inhibitory activity of *L. innocua* isolates

Three patterns of inhibition were noted for the isolates tested and could be described as follows: (i) no inhibition, (ii) zone of inhibition approximately equal to the size of the original colony, and (iii) zone of inhibition that extended past the border of the original colony. Both types of zones were well defined: mostly clear and with well-defined borders. These zones were similar to those reported previously *(14, 17)*.

A total of 100 *L. innocua* and *L. monocytogenes* isolate pairs were screened. Of the 10 *L. innocua* isolates screened, 50% had inhibitory activity against one or more *L. monocytogenes* isolates (Table 3). Two *L. innocua* isolates (Ln-004 and Ln-009) produced

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inhibitory zones that extended beyond the border of the original colony, and three *L. innocua* isolates (Ln-002, Ln-003, and Ln-010) produced inhibitory zones that were approximately equal to the size of the original colony (Table 3).

Sensitivity to the inhibitory activity of the *L. innocua* isolates was typically unitary, i.e., all *L. monocytogenes* test isolates were either sensitive or all were resistant, with two notable exceptions. Isolate Lm-027 was resistant to the inhibitory activity of *L. innocua* isolates Ln-002 and Ln-004, but the other nine *L. monocytogenes* isolates were sensitive. *L. innocua* isolate Ln-009 inhibited only one *L. monocytogenes* isolate (Lm-077). The specific type of inhibitory activity was not addressed in this study; however, others have reported the growth inhibition of *L. monocytogenes* by *L. innocua* resulting from the production of bacteriocins, bacteriophage, or defective bacteriophage particles *(14)*.

Recovery of *L. monocytogenes* by the streak plate method

The initial spiking levels as determined by plate count were 32 to 184 CFU/g for *L. monocytogenes* and 106 to 158 CFU/g for *L. innocua*. The streak plate isolation method was used to obtain individual colonies on Oxoid chromogenic *Listeria*, PALCAM, and Oxford agar plates. When chromogenic *Listeria* agar was used for colony isolation, *L. monocytogenes* was not detected in 18 of 30 enrichment cultures (Table 4). Five *L. monocytogenes* isolates (Lm-002, Lm-003, Lm-004, Lm-006, and Lm-010) were not detected in all enrichments in which they were included. Fractional recovery was observed for two of the isolates (Lm-001 and Lm-039), and three isolates (Lm-027, Lm-051, and Lm-077) were recovered in all enrichments in which they were included. When PALCAM agar was used for colony isolation, *L. monocytogenes* was not detected in 8 of 30 enrichments (Table 4). No individual isolates went completely undetected in all enrichments in which they were included. Fractional recovery was observed for five of the isolates (Lm-001, Lm-002, Lm-004, Lm-006, and Lm-039). Five isolates (Lm-003, Lm-010, Lm-027, Lm-051, and Lm-077) were recovered in all enrichments in which they were included. When Oxford agar was used for colony isolation, *L. monocytogenes* was not detected in 16 of 30 enrichments (Table 4). Five isolates (Lm-001, Lm-002, Lm-003, Lm-004, and Lm-006) were not detected in all enrichments in which they were included. Fractional recovery was observed for one isolate (Lm-039), and four isolates (Lm-010, Lm-027, Lm-051, and Lm-077) were recovered in all enrichments in which they were included.

The presence of *L. innocua* reduced the recovery of *L. monocytogenes* from 48-h enrichments of artificially inoculated guacamole. Overgrowth of *L. innocua* completely masked the presence of *L. monocytogenes* in 7 of 30 enrichments regardless of the isolation medium used. For several enrichments, only 1 or 2 of the 10 colonies selected for confirmation were identified as *L. monocytogenes*, e.g., for Lm-001 grown on *Listeria* chromogenic media only 1 of 30 colonies was confirmed to be *L. monocytogenes* (Table 4). Lm-027 was least susceptible to inhibition by *L. innocua*; all enrichments of this pair were positive for *L. monocytogenes*, and more than half of the colonies recovered were confirmed as *L. monocytogenes*.

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What is the significance of interspecies inhibitory activity during enrichment?

The ability to elicit an inhibitory response and the sensitivity to inhibitory substances among the various species of *Listeria* has been well established *(14, 17)*. However, the effect of this inhibitory effect on the recovery of *L. monocytogenes* during standard culture-based enrichments remains unclear. The deferred antagonism plate assay was used successfully in this study to detect two types of inhibitory activity by *L. innocua* against *L. monocytogenes*. Of the 10 *L. innocua* isolates used in this study, 50% were capable of inhibiting growth of at least 1 of the 10 *L. monocytogenes* isolates. Regarding their sensitivity to the inhibitory effect of *L. innocua*, the *L. monocytogenes* strains used in this study appear similar to other strains previously reported, thus eliminating bias that could result from the inclusion of strains with unusually high or low sensitivity.

The detection of inhibitory activity of *L. innocua* against *L. monocytogenes* during competitive growth appears to be fairly common, particularly when a larger numbers of isolates are screened *(14, 17)*. However, the frequency of inhibition is not high enough by itself to make a determination of whether inhibitory activity can affect the recovery of *L. monocytogenes* from food sample enrichments. Lm-002 had the shortest generation time of the 10 *L. monocytogenes* isolates used in this study, and its generation time was similar to those of Ln-002 (inhibitory *L. innocua* strain) and Ln-005 (noninhibitory *L. innocua* strain) (Table 1). When coinoculated at equivalent levels and grown in BLEB, Ln-002 outgrew Lm-002 by 2.1 log CFU/ml (Table 2). When this same *L. monocytogenes* isolate was paired with Ln-005, the population differential was decreased to only 0.5 log CFU/ml, although *L. innocua* was still the dominant species (Table 2). This finding suggests that growth rate and subsequent nutrient depletion alone may not be sufficient to explain the competitive advantage that *L. innocua* frequently displays over *L. monocytogenes* during standard enrichment procedures. To investigate further, a statistical comparison was made of the population differentials between *L. innocua* strains with inhibitory activity and strains without. The population differentials for both of these groups were not normally distributed and thus were analyzed by the Mann-Whitney rank sum test. The median population differentials were −1.9 and −1.3 log CFU/ml for inhibitory strains and noninhibitory strains of *L. innocua*, respectively, and the difference between these two groups was significant ($P = 0.003$).

Lm-002 was sensitive to the presence of some but not all *L. innocua* isolates screened with the deferred antagonism plate assay (Table 3). When Lm-002 was grown with noninhibitory isolates of *L. innocua* (Ln-001, Ln-005, Ln-006, Ln-007, Ln-008, and Ln-009) the mean population differential was 0.9 ± 0.2 log CFU/ml. When Lm-002 was grown in the presence of inhibitory isolates of *L. innocua* (Ln-002, Ln-003, Ln-004, and Ln-010), the mean population differential was −1.6 ± 0.7 log CFU/ml. The difference between the inhibitory and noninhibitory pairings (−0.9 and −1.6 log CFU/ml) was not significant ($P = 0.07$) at the 95% confidence level. The population differential for the Lm-002 and Ln-010 pair (Table 2) was less than what would be expected based upon the results of the deferred antagonism plate assay (Table 3). This low value could be an indication that different levels of sensitivity to the inhibitory activity of this particular *L. innocua* isolate were initially not detected because of the qualitative nature of the deferred antagonism plate assay. When the datum for

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this pair is removed from the analysis, then the mean population difference between the remaining three inhibitory isolates of *L. innocua* (Ln-002, Ln-003, and Ln-004) and Lm-002 is −2.0 ± 0.2 log CFU/ml, and the difference in mean population differentials between the inhibitory and noninhibitory pairings (−0.9 and −2.0 log CFU/ml) is significant ($P \leq 0.001$).

What is the significance of growth rate on enrichment populations and recovery of *L. monocytogenes*?

In 98 of 100 paired isolates, *L. innocua* reached higher levels by 48 h than did *L. monocytogenes* when grown in BLEB at 30°C (Table 2). However, in only 39 of the 100 pairs was the presence of inhibitory activity indicated by the results of the deferred antagonism plate assay (Table 3). This finding suggests that simple differences in growth rates and the resulting effects of nutrient depletion by the more rapidly growing isolate may account for the majority of reported incidences of *L. innocua* outcompeting *L. monocytogenes* during standard enrichment procedures. A more quantitative assessment of the effects of growth rate can be made by comparing the 48-h population differentials between noninhibitory isolates of *L. innocua* and *L. monocytogenes* Lm-002 and Lm-051, which have the shortest and longest generation times, respectively (Table 1). When Lm-002 was grown with noninhibitory isolates of *L. innocua* (Ln-001, Ln-005, Ln-006, Ln-007, Ln-008, and Ln-009), the overall mean difference in the populations between the two species was −0.9 ± 0.2 log CFU/ml. When Lm-051 was grown with these same *L. innocua* isolates, the overall mean difference in the populations between the two species was −1.5 ± 0.2 log CFU/ml. Table 2 shows the difference in the populations between *L. innocua* and *L. monocytogenes* when simultaneously inoculated and grown for 48 h in BLEB. When the population differentials for Lm-002 and Lm-051 were directly compared with each noninhibitory L. innocua isolate, a 0.2- to 1-log (with an average of 0.6 ± 0.3 log CFU/ml) increase in these 48-h enrichment population differentials was found for the isolate with the longer generation time.

In contrast, the postenrichment population differentials resulting from pairings of these same two *L. monocytogenes* isolates with inhibitory *L. innocua* isolates were not as large (Table 2). The population differentials for Lm-002 and Lm-051, which have the shortest and longest generation times, respectively, were essentially identical when each was paired with the inhibitory Ln-002, Ln-004, and Ln-010. The final enrichment populations for these particular pairs were more heavily influenced by the inhibitory activity of *L. innocua* than by simple differences in growth rate. When these two *L. monocytogenes* isolates were further compared with Ln-003, the other inhibitory isolate, a 0.5-log difference in the enrichment population differentials was found. The 48-h enrichment population of Lm-051 was more inhibited by the presence of Ln-003 than was the population of Lm-002. This finding can be explained also by a difference in sensitivity of the *L. monocytogenes* isolates to the inhibitory activity of *L. innocua*. Growth rate and inhibitory activity do not appear to have additive effects, and the final populations of each species following enrichment are likely to be more heavily influenced by one factor or the other.

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Effect of preenrichment species levels

L. monocytogenes CFSAN-82 at 2.7 to 0.7 log CFU/ml was simultaneously inoculated with Ln-001 at 2.8 to 0.8 log CFU/ml into BLEB (Table 5). After 48 h of incubation at 30uC, the total *Listeria* population as determined by surface plating onto Oxford agar was 9.1 ± 0.04 log CFU/ml and did not appear to fluctuate when the initial inoculum level of either species was changed. The 48-h enrichment population of *L. monocytogenes* was highly dependent on the spiking levels. A 1-log decrease from 2.7 to 1.7 log CFU/ml in the *L. monocytogenes* spiking level resulted in an approximately 0.9-log reduction in the 48-h enrichment population when the levels of *L. innocua* were held constant at 0.8 log CFU/ ml (Table 5). An additional 1.2-log reduction was observed when the *L. monocytogenes* spiking level was further decreased from 1.7 to 0.7 log CFU/ml. A similar trend was observed when the spiking levels of *L. monocytogenes* were held constant at 0.7 log CFU/ml and those of *L. innocua* were varied (Table 5). A decrease in the initial level of *L. innocua* from 2.8 to 1.8 log CFU/ml yielded an approximate 0.8-log increase in the 48-h enrichment population of *L. monocytogenes*. When the spiking level of *L. innocua* was further decreased to 0.8 log CFU/ml, an additional 0.8-log increase in the 48-h enrichment population of *L. monocytogenes* was again observed.

The preenrichment levels of *L. innocua* also affected the recovery of *L. monocytogenes* with the streak plating method (Table 5). *L. monocytogenes* was recovered and confirmed consistently only when it was initially present at the highest inoculum level used in this study. Under these conditions, *L. monocytogenes* was recovered in all three replicate enrichments using both isolation media. At least twice as many total colonies were confirmed as *L. monocytogenes* when colonies were isolated on Oxford agar. The use of Oxoid chromogenic *Listeria* agar did not aid in colony selection even when *L. monocytogenes* and *L. innocua* were present at approximately equal levels. One possible reason lack of an effect is insufficient levels of phosphatidylinositol phospholipase C activity by *L. monocytogenes* CFSAN-82 to allow for differentiation between this strain and *L. innocua* even after the plates were incubated a total of 72 h.

All isolates, including CFSAN-82, were spot inoculated (5 μl from a 24-h culture) onto the surface of the chromogenic *Listeria* agar. Well-defined halos surrounding the colonies were easily visualized within 24 h at 37°C. However, the resulting colonies from a 5-μl spot inoculation are much larger and likely produce considerably more phospholipase C activity than colonies originating from individual cells as is presumed the case with streak plating detection. Limitations on colony size induced by crowding on the surface of the plate, particularly by *Listeria* species other than *L. monocytogenes*, can limit the usefulness of phospholipase C differentiation. When the spiking level of *L. monocytogenes* was reduced from 2.7 to 1.7 log CFU/ml, this species was no longer recoverable on Oxford agar. All three enrichment replications were still confirmed as positive when chromogenic *Listeria* agar was used, with a total of nine colonies testing positive. Although the enumeration studies indicated only a 1.1-log difference between *L. innocua* and *L. monocytogenes* enrichment populations, *L. monocytogenes* was not detected with Oxford agar. *L. monocytogenes* was not recovered with any of the other inoculation scenarios used in this study.

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This work was conducted to investigate the competition between *L. monocytogenes* and *L. innocua* during 48 h of enrichment culture in BLEB at 30°C. In a majority of the isolate pairs, *L. innocua* outcompeted *L. monocytogenes* sometimes by as much as 2 log CFU/ml, hindering recovery of the pathogenic species by streak plate isolation. The effects of growth rate of both species and the presence of the inhibitory activity of certain *L. innocua* isolates was also investigated. The final enrichment population of each species was determined by either a shorter generation time or the presence of inhibitory activity. These two factors did not appear additive; either one or the other was more influential in each of the isolate pairs. The presence of multiple *Listeria* species in foods is routinely encountered; thus, *L. innocua* poses a particular problem because it can mask the presence of *L. monocytogenes*. Continued improvements in *L. monocytogenes* isolation methods are needed to reduce the chances of false-negative results due to the presence of other *Listeria* species.

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TABLE 1

Generation time (GT), final population after growth in enrichment broth, and isolate source of L. monocytogenes *4b and* L. innocua *isolates used in this study*

Isolate[a]	GT (h)	48-h population (log CFU/ml)[b]	Source
ARL-Lm-001	1.8 ± 0.1	8.9 ± 0.1	Imported cold smoked salmon
ARL-Lm-002	1.5 ± 0.1	9.0 ± 0.1	Imported Mexican style cheese
ARL-Lm-003	2.3 ± 0.1	9.2 ± 0.1	Imported Mexican style cheese
ARL-Lm-004	2.0 ± 0.0	9.2 ± 0.1	Imported Mexican style cheese
ARL-Lm-006	1.7 ± 0.1	9.1 ± 0.1	Salmon
ARL-Lm-010	1.9 ± 0.1	9.1 ± 0.1	Ricotta salata cheese
ARL-Lm-027	1.6 ± 0.1	9.0 ± 0.2	Mexican style cheese
ARL-Lm-039	2.0 ± 0.1	9.2 ± 0.1	Mexican style cheese
ARL-Lm-051	2.4 ± 0.1	9.0 ± 0.1	Frozen cooked shrimp
ARL-Lm-077	1.7 ± 0.1	8.9 ± 0.1	Environmental swab (cheese manufacturing)
CFSAN-82	1.6 ± 0.1	8.9 ± 0.1	CFSAN culture collection
ARL-Ln-001	1.7 ± 0.1	9.3 ± 0.1	Imported fresh crawfish
ARL-Ln-002	1.4 ± 0.1	9.2 ± 0.1	Imported seafood salad
ARL-Ln-003	1.0 ± 0.1	9.3 ± 0.1	Imported cooked fried rice
ARL-Ln-004	1.2 ± 0.1	9.2 ± 0.1	Mozzarella cheese
ARL-Ln-005	1.4 ± 0.1	9.2 ± 0.1	Imported Mexican style cheese
ARL-Ln-006	1.2 ± 0.1	9.3 ± 0.1	Imported Mexican style cheese
ARL-Ln-007	1.2 ± 0.1	9.2 ± 0.1	Environmental swab (spice blending)
ARL-Ln-008	1.0 ± 0.1	9.2 ± 0.1	Imported fresh crawfish
ARL-Ln-009	0.9 ± 0.1	9.2 ± 0.1	Environmental swab (cheese manufacturing)
ARL-Ln-010	1.1 ± 0.1	9.1 ± 0.1	Environmental swab (sandwich manufacturing)

[a] Lm, *L. monocytogenes*; Ln, *L. innocua*. CFSAN-82 is a streptomycin-resistant variant of *L. monocytogenes* Scott A.

[b] Population after 48 h at 30°C in BLEB with selective supplements.

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TABLE 2

L. monocytogenes (*Lm*) and L. innocua (*Ln*) *postenrichment population differentials*

	Mean (SD) population differentials (log CFU/ml)[a]									
L. innocua	**Lm-001**	**Lm-002**	**Lm-003**	**Lm-004**	**Lm-006**	**Lm-010**	**Lm-027**	**Lm-039**	**Lm-051**	**Lm-077**
Ln-001	−1.4 (0.3)	−1.0 (0.2)	−1.0 (0.2)	−1.7 (0.1)	−0.8 (0.1)	−1.2 (0.2)	−1.2 (0.3)	−0.8 (0.2)	−1.4 (0.1)	−0.6 (0.2)
Ln-002	−2.1 (0.2)	−2.1 (0.1)	−2.1 (0.2)	−2.2 (0.1)	−2.1 (0.2)	−1.8 (0.3)	−2.1 (0.1)	−2.4 (0.2)	−2.1 (0.3)	−1.5 (0.3)
Ln-003	−1.9 (0.1)	−1.7 (0.1)	−1.7 (0.3)	−2.1 (0.1)	−1.2 (0.3)	−1.7 (0.3)	−1.4 (0.2)	−2.0 (0.2)	−2.2 (0.1)	−1.7 (0.4)
Ln-004	−1.3 (0.3)	−2.2 (0.1)	−2.1 (0.1)	−1.4 (0.2)	−2.2 (0.1)	−2.2 (0.2)	−1.8 (0.4)	−2.3 (0.1)	−2.3 (0.1)	−1.4 (0.5)
Ln-005	−2.4 (0.2)	−0.5 (0.1)	−0.8 (0.1)	−1.2 (0.1)	−0.2 (0.1)	−0.9 (0.2)	−1.4 (0.1)	−1.5 (0.1)	−1.5 (0.2)	−1.0 (0.1)
Ln-006	−1.8 (0.4)	−1.1 (0.1)	−2.2 (0.2)	−1.8 (0.2)	−1.0 (0.1)	−1.3 (0.4)	−2.4 (0.1)	−1.6 (0.3)	−1.3 (0.2)	−0.8 (0.1)
Ln-007	−2.1 (0.2)	−0.9 (0.1)	−1.9 (0.4)	−1.8 (0.1)	−1.0 (0.1)	−1.3 (0.1)	−1.7 (0.4)	−0.7 (0.1)	−1.6 (0.2)	−0.4 (0.1)
Ln-008	−2.1 (0.4)	−0.9 (0.1)	−1.9 (0.4)	−1.6 (0.1)	−0.9 (0.1)	−1.5 (0.1)	−1.6 (0.2)	−0.5 (0.1)	−1.2 (0.1)	−0.2 (0.1)
Ln-009	−2.3 (0.1)	−1.2 (0.1)	−2.2 (0.2)	−2.2 (0.1)	−2.0 (0.4)	−2.2 (0.2)	−2.3 (0.1)	−1.2 (0.1)	−1.9 (0.1)	−1.0 (0.1)
Ln-010	−2.1 (0.1)	−0.5 (0.1)	−1.9 (0.2)	−1.9 (0.1)	−0.5 (0.1)	−0.3 (0.1)	0.3 (0.4)	−0.2 (0.2)	−0.5 (0.3)	0.4 (0.2)

[a]Differential determined as *L. monocytogenes* – *L. innocua*. Values are mean (SD) of three replications.

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TABLE 3

Inhibitory activity of L. innocua *(Ln) against* L. monocytogenes (Lm) determined by the deferred antagonism plating assay

	Inhibition of Lm by Ln[a]									
L. innocua	**Lm-001**	**Lm-002**	**Lm-003**	**Lm-004**	**Lm-006**	**Lm-010**	**Lm-027**	**Lm-039**	**Lm-051**	**Lm-077**
Ln-001	−	−	−	−	−	−	−	−	−	−
Ln-002	+	+	+	+	+	+	−	+	+	+
Ln-003	+	+	+	+	+	+	+	+	+	+
Ln-004	++	++	++	++	++	++	−	++	++	++
Ln-005	−	−	−	−	−	−	−	−	−	−
Ln-006	−	−	−	−	−	−	−	−	−	−
Ln-007	−	−	−	−	−	−	−	−	−	−
Ln-008	−	−	−	−	−	−	−	−	−	−
Ln-009	−	−	−	−	−	−	−	−	−	++
Ln-010	+	+	+	+	+	+	+	+	+	+

[a]Original colony size was 11.3 ± 0.6 mm.

−no inhibition zone observed;

+zone of clearing (13.7 ± 1.1 mm) approximately equal to the size of the original colony;

++zone of clearing (20.2 ± 1.5 mm) extending beyond the original colony border.

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TABLE 4

Isolation of L. monocytogenes *(Lm) from mixed culture with* L. innocua ARL-Ln-010 after 48 h of enrichment of artificially inoculated guacamole on various selective and/or differential media

	No. of Lm-postive enrichment cultures and colonies/no. tested[a]										
Culture type	**Lm-001**	**Lm-002**	**Lm-003**	**Lm-004**	**Lm-006**	**Lm-010**	**Lm-027**	**Lm-039**	**Lm-051**	**Lm-077**	**Total**
Chromogenic agar											
Enrichments	1/3	0/3	0/3	0/3	0/3	0/3	3/3	2/3	3/3	3/3	12/30
Colonies	1/30	0/30	0/30	0/30	0/30	0/30	17/30	2/30	8/30	6/30	34/300
PALCAM agar											
Enrichments	1/3	2/3	3/3	1/3	2/3	3/3	3/3	1/3	3/3	3/3	22/30
Colonies	1/30	2/30	4/30	1/30	3/30	6/30	23/30	2/30	10/30	9/30	61/300
Oxford agar											
Enrichments	0/3	0/3	0/3	0/3	0/3	3/3	3/3	2/3	3/3	3/3	14/30
Colonies	0/30	0/30	0/30	0/30	0/30	5/30	24/30	3/30	13/30	12/30	57/300
Total											
Enrichments	2/9	2/9	3/9	1/9	2/9	6/9	9/9	4/9	9/9	9/9	
Colonies	2/90	2/90	4/90	1/90	3/90	11/90	64/90	7/90	31/90	27/90	

[a]Three enrichment cultures per isolate pair were tested. Ten colonies per enrichment culture were selected for confirmation, for a total of 30 colonies tested.

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TABLE 5

Influence of initial L. monocytogenes *CFSAN-82 (Lm) and* L. innocua ARL-Ln-001 (Ln) spiking levels on recovery and final 48-h populations of L. monocytogenes *in BLEB at 30°C*

Results	Mean ± SD initial spiking levels (log CFU/ml)[a]:				
	Lm: 2.7 ± 0.06 Ln: 0.8 ± 0.01	**Lm: 1.7 ± 0.06 Ln: 0.8 ± 0.01**	**Lm: 0.7 ± 0.06 Ln: 0.8 ± 0.01**	**Lm: 0.7 ± 0.06 Ln: 1.8 ± 0.01**	**Lm: 0.7 ± 0.06 Ln: 2.8 ± 0.01**
48-h enrichment population (log CFU/ml)[b]					
L. monocytogenes	9.0 ± 0.10	8.1 ± 0.07	6.9 ± 0.13	6.1 ± 0.09	5.3 ± 0.2
Listeria spp.	9.1 ± 0.05	9.2 ± 0.03	9.1 ± 0.03	9.1 ± 0.05	9.1 ± 0.07
Streak plate recovery of *L. monocytogenes*[c]					
Oxford agar					
Colonies	24/30	0/30	0/30	0/30	0/30
Replicates	3/3	0/3	0/3	0/3	0/3
Chromogenic *Listeria* agar					
Colonies	9/30	5/30	0/30	0/30	0/30
Replicates	3/3	3/3	0/3	0/3	0/3

[a] Spiking levels were estimated by plate count on TSAYE following serial dilution of the overnight culture in phosphate buffer.

[b] *L. monocytogenes* populations were determined on TSAYE supplemented with 200 μg/ml streptomycin sulfate. Total *Listeria* spp. populations were determined by surface plating onto Oxford agar.

[c] Streak plate analysis was performed using both Oxford agar and Oxoid chchromogenic *Listeria* agar. Results are expressed qualitatively as the number of positive colonies or replicates/number tested.