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# Bias in the *Listeria monocytogenes* Enrichment Procedure: Lineage 2 Strains Outcompete Lineage 1 Strains in University of Vermont Selective Enrichments

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***Listeria monocytogenes*** **can be isolated from a range of food products and may cause food-borne outbreaks or sporadic cases of listeriosis. *L. monocytogenes* is divided into three genetic lineages and 13 serotypes. Strains of three serotypes (1/2a, 1/2b, and 4b) are associated with most human cases of listeriosis. Of these, strains of serotypes 1/2b and 4b belong to lineage 1, whereas strains of serotype 1/2a and many other strains isolated from foods belong to lineage 2. *L. monocytogenes* is isolated from foods by selective enrichment procedures and from patients by nonselective methods. The aim of the present study was to investigate if the selective enrichment procedure results in a true representation of the subtypes of *L. monocytogenes* present in a sample. Eight *L. monocytogenes* strains (four lineage 1 strains and four lineage 2 strains) and one *Listeria innocua* strain grew with identical growth rates in the nonselective medium brain heart infusion (BHI), but differed in their growth rate in the selective medium University of Vermont medium I (UVM I). When coinoculated in UVM I, some strains completely outgrew other strains. This outcome was dependent on the lineage of *L. monocytogenes* rather than the individual growth rate of the strains. When inoculated at identical cell densities in UVM I, *L. innocua* outcompeted *L. monocytogenes* lineage 1 strains but not lineage 2 strains. In addition, lineage 2 *L. monocytogenes* strains outcompeted lineage 1 *L. monocytogenes* strains in all combinations tested, indicating a bias in strains selected by the enrichment procedures. Bias also occurred when coinoculating two lineage 2 or lineage 1 strains; however, it did not appear to correlate with origin (clinical versus food). Identical coinoculation experiments in BHI suggested that the selective compounds in UVM I and II influenced this bias. The results of the present study demonstrate that the selective procedures used for isolation of *L. monocytogenes* may not allow a true representation of the types present in foods. Our results could have a significant impact on epidemiological studies, as lineage 1 strains, which are often isolated from clinical cases of listeriosis, may be suppressed during enrichment by other *L. monocytogenes* lineages present in a food sample.**

*Listeria monocytogenes* is a food-borne pathogenic bacterium which can cause outbreaks or sporadic cases of listeriosis. The incidence of listeriosis is low; however, the bacterium is very important for food safety, since listeriosis is associated with very high mortality, approximately 25%, in susceptible individuals (9). It is crucial for implementation of control measures in the food industry that the food sources causing listeriosis be identified at points in the food chain where contamination occurs and that potential differences in virulence among different subtypes be understood. To be able to perform such analyses, subtyping of *L. monocytogenes* is crucial and is primarily based on serotyping or molecular methods (35). *L. monocytogenes* is divided into 13 known serovars, of which especially three, 4b, 1/2a, and 1/2b, are isolated from cases of listeriosis (20). Nucleotide variation in three virulence genes (*actA*, *inlA*, *hly*) grouped strains of *L. monocytogenes* into three groups (lineage 1, 2, and 3) (28, 36). The majority of clinical strains were found within lineage 1, which covers serotypes 4b and 1/2b, whereas serotype 1/2a belongs to lineage 2 (21). No clinical isolates were found within lineage 3 (36).

*Listeria monocytogenes* is commonly isolated from many different types of foods, including ready-to-eat products. Listeriosis is primarily associated with such products as they are not subjected to thermal treatment before consumption and an extended refrigerated shelf life may allow growth of the microorganism. The bacterium is often isolated from food-processing environments, where it may persist and cause continuous contamination of food products (11, 31). Recently, it has been suggested that biofilm formation by *L. monocytogenes* and, hence, potentially its ability to persist may differ among strains of different lineages (1).

Detection of *L. monocytogenes* from foods and food processing environments can be difficult because the bacterium in contaminated foods is normally found in very low numbers in a heterogeneous microflora. Hence, as is common practice for a number of food-borne pathogenic bacteria, enrichment steps are included in the analyses, and detection is often limited by the performance of the enrichment broth. Several studies have evaluated the performance of different isolation methods for their ability to detect low levels of *L. monocytogenes*, as well as injured cells (4, 25, 32, 33). One of the most commonly used enrichment broths is the University of Vermont medium (UVM), which contains nalidixic acid (suppresses gram-negative bacteria) and acriflavin (suppresses gram-positive bacteria) as selective supplements. Ideally, such enrichment procedures and selective medium should allow only the target organisms to proliferate. Also, since *L. monocytogenes* is often

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isolated from foods as part of epidemiological investigations, the procedures for enrichment and detection should ensure that all subtypes present in a sample be represented after enrichment. However, when sampling from foods, Loncarevic et al. (17) found more *L. monocytogenes* clones by direct plating compared to isolation following enrichment procedures.

Other *Listeria* species such as *Listeria innocua* are often found in foods, and since *L. innocua* may have a faster growth rate than *L. monocytogenes* in UVM and other enrichment broths, *L. innocua* may overgrow *L. monocytogenes*, which then escapes detection (6, 18, 26). Such observations also raise the question of whether the enrichment procedures allow equal growth of all subtypes of *L. monocytogenes*. If not, the enrichment procedure may be biased in selecting specific strains and subsequent epidemiological studies may be seriously hampered. Gracieux et al. (13) concluded that virulent *L. monocytogenes* strains reached significantly higher cell counts on selective agar media such as PALCAM, Oxford, Rapid L. mono (RLM), and ALOA Listeria agar than did nonvirulent strains. However, their study did not address any biases of these enrichment procedures.

Any systematic bias in the enrichment procedure vis-a-vis subtypes will clearly have a major impact on epidemiological studies. This is true in cases where food sources causing outbreaks are traced as well as studies of contamination of food-processing environments. In clinical investigations, *L. monocytogenes* will typically be isolated from blood or cerebrospinal fluid samples either directly on nonselective plates or following enrichment in nonselective broths. Obviously, the clinical isolates which are compared to food isolates have been isolated following a very different procedure. A number of studies have attempted to determine the links between specific ready-to-eat products and listeriosis by comparing large collections of *L. monocytogenes* isolated from foods with strains from clinical cases. Subtyping large collections of isolates by combinations of different molecular subtyping methods (pulsed-field gel electrophoresis, ribotyping, and randomly amplified polymorphic DNA) have not revealed any links between specific foods and disease (12, 19).

Based on the above, the purpose of the present study was to determine if one of the most common enrichment procedures for *L. monocytogenes* is biased in terms of differentially selecting specific subgroups. Also, the competition between *L. innocua* and *L. monocytogenes* during enrichment in UVM was investigated.

## MATERIALS AND METHODS

**Bacterial strains and storage conditions.** Eight strains of *L. monocytogenes* and one strain of *L. innocua* were used in the experiments (Table 1). The strains were chosen to allow lineages 1 and 2 to be represented by both food isolates and clinical strains. Six of the eight strains and the *L. innocua* strain were chosen as representing the major clusters described by Fonnesbech Vogel et al. (10). Cultures were stored at −80°C and streaked on brain heart infusion (BHI) plates (Oxoid CM225, Oxoid Ltd. Basingstoke, Hampshire, United Kingdom) and incubated for 24 h at 37°C before further culturing.

**Growth rate of individual *Listeria* strains in UVM and BHI.** To determine if any possible selection in the selective University of Vermont medium (UVM) (Oxoid CM863 supplemented with SR0142 or SR0143) enrichment medium is caused by different growth rates, the growth rate and maximum cell density of all strains were determined in UVM I. One colony from each strain was inoculated into 10 ml of BHI broth and incubated for 24 h at 37°C. The cultures were diluted in BHI to a cell density of approximately $10^8$ CFU/ml. Cell densities were adjusted based on optical density at 450 nm and standard curves relating colony counts to optical density were constructed for each strain.

TABLE 1. *Listeria* strains used in this study

| Species | Serogroup | Lineage | Strain | Origin | Reference |
|---|---|---|---|---|---|
| *L. monocytogenes* | 1/2a | 2 | La22 | Salmon smokehouse | 11 |
| | 1/2a | 2 | HU4239 | Clinical case | 36 |
| | 1/2a | 2 | V5a | Salmon smokehouse | 11 |
| | 1/2a | 2 | C1-056 | Clinical case | 36 |
| | 4b | 1 | V518a | Salmon smokehouse | 11 |
| | 4b | 1 | 4542 | Clinical case | 11 |
| | 4b | 1 | C1-109 | Clinical case | 36 |
| | 1/2b | 1 | 7418 | Sausage | 11 |
| *L. innocua* | | | R255a | Salmon smokehouse | 11 |

The bacterial cultures were inoculated in 250 ml of UVM I (Oxoid CM863) at an initial level of 40 CFU/ml. Initial cell densities were confirmed by spread plating of the inoculation culture on BHI agar (incubated for 24 h at 37°C). Growth in UVM I at 30°C for 24 h was followed by sampling every 4 h and determining cell densities by spread plating on BHI (incubated for 24 h at 37°C); 100 μl of 24-h UVM I culture was transferred to 9.9 ml of UVM II and incubated for 24 h at 30°C, after which cell densities were determined by plate counts on BHI (incubated for 24 h at 37°C). Growth rates were determined from the exponential part of the growth curve (typically $10^2$ to $10^7$ CFU/ml) and comparisons of growth rates were done with Student's *t* test. Also, the maximum cell densities after growth for 24 h at 30°C in BHI were determined for all strains following an inoculation of approximately 40 CFU/ml.

**Competition between *Listeria* strains in UVM I and II and BHI.** All strains were tested in selected combinations, allowing mixtures of *L. monocytogenes* and *L. innocua* as well as mixtures of different serotypes or lineages of *L. monocytogenes* from different sources to be studied. Cell densities of the precultures, grown as monocultures, were adjusted, reaching an initial cell density of 40 CFU/ml. After incubation of UVM I at 30°C for 24 h, 0.1 ml of the UVM I culture was transferred to 9.9 ml of UVM II (Oxoid CM863) and incubated for a further 24 h at 30°C. The density of *L. monocytogenes* and *L. innocua* in mixed cultures was determined by spread plating on Rapid L. mono (RLM) (Bio-Rad, Ivry-sur-Seine, France), where *L. monocytogenes* appears as blue colonies and *L. innocua* as white colonies.

To ensure that the selective plating medium itself did not bias the counting, these mixed cultures were also spread plated onto blood agar plates (incubated for 24 h at 37°C), where *L. monocytogenes* colonies appeared with zones of hemolysis whereas *L. innocua* was nonhemolytic. Cell densities of mixtures of two *L. monocytogenes* strains were determined by spread plating on BHI-agar, and to determine the ratio of the two strains, 34 to 40 colonies were isolated from the BHI plates and each was inoculated in 5 ml of BHI for 24 h at 37°C. Discrimination between the two *L. monocytogenes*, strains were done with serotyping and/or randomly amplified polymorphic DNA typing of the individual isolates (see below). Competition experiments within BHI were conducted as for UVM I.

**Serotyping and lineage determination.** Serotyping was performed with commercial O-antigen *Listeria* antisera one and four (Bacto Listeria O antiserum 1 223001 and Bacto Listeria O antiserum 4 223011; Difco Laboratories). Colonies were grown in BHI broth for 24 h, and 1.5 ml of culture was boiled for 1 h. The samples were centrifuged at approximately 9,500 × *g* for 2 min, and the supernatant was removed. The pellets were resuspended in the remaining liquid. One drop of the resuspended organisms was mixed with one drop of antiserum on a glass plate and the plate was gently rocked for 1 min. Positive reaction was seen as coagulation in the sample. All samples were tested with both antisera one and four. The differentiation between serotypes a and b was done by Health Canada. The division in lineage 1 and 2 strains was derived from Fonnesbech Vogel et al. (10).

**Randomly amplified polymorphic DNA typing.** DNA purification and randomly amplified polymorphic DNA (RAPD) were performed as previously described (11). Isolates were grown for 24 h in BHI at 37°C and DNA was purified with Dynabeads Dynal direct system 1 (Dynal A/S & Nordic, Oslo, Norway). Randomly amplified polymorphic DNA (RAPD) amplification was carried out with Ready-To-Go RAPD analysis beads (Amersham Pharmacia Biotech, Inc., Piscataway, N.J.) and primer HLWL85 (5′-ACAACTGCTC; DNA Technology,

TABLE 2. Growth of six *Listeria monocytogenes* strains and one *Listeria innocua* strain in brain heart infusion broth and in UVM I and II

| Species | Strain | Log cell density after 24 h (CFU/ml) | | | Generation time (h) in UVM I[c] |
|---|---|---|---|---|---|
| | | BHI[a] | UVM I[a] | UVM II[b] | |
| *L. monocytogenes* | La22 | 9.70 ± 0.15 | 7.59 ± 0.27 | 8.98 ± 0.19 | 1.06 ± 0.05[a,b] |
| | Hu4239 | 9.76 ± 0.12 | 7.64 ± 0.20 | 8.62 ± 0.29 | 1.09 ± 0.03[a,b] |
| | V5a | 9.81 ± 0.05 | 7.21 ± 0.14 | 9.11 ± 0.04 | 1.18 ± 0.09[b,c,d] |
| | C1-056 | 9.80 ± 0.04 | 6.72 ± 0.71 | 8.97 ± 0.06 | 1.28 ± 0.15[c,d,e] |
| | V518a | 9.77 ± 0.10 | 7.78 ± 0.21 | 9.05 ± 0.01 | 1.05 ± 0.02[a] |
| | C1-109 | 9.80 ± 0.21 | 6.22 ± 0.38 | 7.74 ± 1.79 | 1.31 ± 0.09[c,e] |
| | 4542 | 9.55 ± 0.15 | 7.04 ± 0.01 | 8.63 ± 0.03 | 1.18 ± 0.02[c,d] |
| | 7418 | 9.42 ± 0.06 | 7.30 ± 0.04 | 8.46 ± 0.05 | 1.16 ± 0.01[c,d] |
| *L. innocua* | R255a | 9.75 ± 0.04 | 7.32 ± 0.81 | 8.98 ± 0.03 | 1.15 ± 0.11[a,c,d,e] |

[a] After growth at 30°C for 24 h at an initial cell density of 40 cells/ml.
[b] When inoculating 100 μl from UVM I into 9.9 ml of UVM II, followed by growth at 30°C for 24 h.
[c] Growth rates with the same roman letter are not statistically significantly different (Student's *t* test at 5% level).

Aarhus, Denmark). The PCR was performed as follows: after denaturing for 2 min at 95°C, the next 10 cycles took place at 1 min of denaturing at 95°C, followed by annealing at 45 to 36°C, reducing the temperature with 1 degree Celsius for each cycle, and followed by 2 min at 72°C. The last 30 cycles used denaturing at 95°C for 1 min followed by annealing at 35°C for 1 min and extension at 72°C for 2 min followed by 10 min at 72°C. Nucleotide bands were separated in 2% agarose run at 90 V for 4 h and were visualized by staining with ethidium bromide.

**Interaction between *Listeria* strains.** The possible antagonistic interaction between *Listeria* strains was determined to evaluate if the differential selection of strains was caused by inhibitory activities. Outgrown cultures as well as sterile filtered supernatants of outgrown cultures from all the *Listeria* spp. grown in BHI or UVM I were tested in an agar well-diffusion bioassay (23) against all of the *Listeria* spp. used in this study. One colony from each *Listeria* spp. was taken from a BHI plate and transferred to 10 ml of BHI broth or 10 ml of UVM I and incubated at 30°C for 24 h. The cell density was adjusted to approximately $10^7$ CFU/ml; 500 μl of culture was inoculated in 50 ml of BHI broth (kept at 46°C) with 0.1% Tween 80, and the culture was immediately poured into plates. Wells (7 mm) were punched in the solidified agar and 50 μl of sterile supernatant or outgrown cultures was added to the wells. The plates were incubated at 30°C for 24 h, and any inhibition was seen as inhibition zones around the wells. Bacteriocin-producing *Carnobacterium piscicola* (23) was used as a positive control.

## RESULTS

**Individual growth.** All *Listeria* species reached almost identical cell densities ($5 \times 10^9$ to $7 \times 10^9$ CFU/ml) when grown in BHI broth (Table 2). In contrast, UVM I limited growth and cell densities were 2 to 3 log10 units lower in UVM I than BHI broth after 24 h. Two clinical isolates (C1-056 and C1-109) were especially sensitive to the selective principles. Subsequent growth in UVM II allowed cultures to reach $10^8$ to $10^9$ CFU/ml (Table 2). Based on these results, the ability of the strains to grow on the selective substrate PALCAM was tested. Identical counts were found when comparing counts on PALCAM and BHI from outgrown cultures of all *Listeria* strains in BHI (data not shown).

***L. innocua* versus *L. monocytogenes* in UVM I and UVM II.** Adjusting inoculation levels in cocultures based on absorbance measurements was very accurate and the inoculum levels of the two bacterial strains were almost identical (Table 3), with an average of 35 CFU/ml (data not shown). When *L. innocua* was coinoculated with *L. monocytogenes* lineage 2 strains, variation in the ratios of *L. monocytogenes* after 24 h of incubation was found, but both species were easily isolated after both UVM I and UVM II culturing. However, *L. monocytogenes* lineage 1 strains were outcompeted by *L. innocua* as only 2 to 6% of the colonies emerging after UVM I selective enrichment were *L. monocytogenes* and virtually no *L. monocytogenes* could be detected after UVM II selection (Table 3). Counts of hemolytic and nonhemolytic colonies on blood-agar plates were identical to counts of blue and white colonies on RLM plates (data not shown), indicating that the selective plates did not bias the counts.

***L. monocytogenes* versus *L. monocytogenes* in UVM I and UVM II.** The very systematic difference in lineage competition ability prompted us to systematically investigate how different *L. monocytogenes* lineages behave in UVM I and UVM II. When lineage 2 and lineage 1 strains were combined, lineage 2 strains outcompeted the lineage 1 strains in all combinations. We isolated a total of 1,208 colonies from combinations of lineage 1 and lineage 2 strains after enrichment in UVM I (32 combinations with approximately 34 to 40 colonies per combination [Table 4]), and 987 colonies (82%) were lineage 2 and 221 colonies (18%) were lineage 1 (data not shown). After enrichment in UVM II, a total of 1,200 colonies were tested

TABLE 3. Proportion of *Listeria monocytogenes* and *Listeria innocua* after coculturing in UVM I and UVM II at 30°C for 24 h[a]

| Lineage | Strains | | No. of independent trials | Inoculation level (%)[b] | | Distribution[c] (%) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | UVM I | | UVM II | |
| | A | B | | A | B | A | B | A | B |
| 2 | La22 | R255a | 3 | 45 | 55 | 20 | 80 | 19 | 81 |
| | | | | 50 | 50 | 35 | 65 | 41 | 59 |
| | | | | 47 | 53 | 50 | 50 | 68 | 32 |
| 2 | V5a | R255a | 3 | 50 | 50 | 9 | 91 | 10 | 90 |
| | | | | 54 | 46 | 14 | 86 | 27 | 73 |
| | | | | 49 | 51 | 77 | 23 | 72 | 28 |
| | | | | 49 | 51 | 72 | 28 | 51 | 49 |
| 2 | C1-056 | R255a | 1 | 50* | 50* | 95 | 5 | 72 | 28 |
| | | | | 50* | 50* | 89 | 11 | 74 | 26 |
| 2 | Hu4239 | R255a | 1 | 51* | 49* | 25 | 75 | 51 | 49 |
| | | | | 51* | 49* | 27 | 73 | 50 | 50 |
| 1 | V518a | R255a | 3 | 49 | 51 | 6 | 94 | 1 | 99 |
| | | | | 42 | 58 | 4 | 96 | 0.3 | 99.7 |
| | | | | 47 | 53 | ND | ND | 1 | 99 |
| 1 | C1-109 | R255a | 1 | 55* | 45* | 3 | 97 | 0 | 100 |
| | | | | 55* | 45* | 2 | 98 | 0 | 100 |

[a] A, *L. monocytogenes*; B, *L. innocua*.
[b] *, inoculations done in duplicate.
[c] Distribution determined by plate counts on Rapid L. mono (RLM). ND, not done

TABLE 4. Proportion of two strains of *Listeria monocytogenes* after coculturing in UVM I and UVM II at 30°C for 24 h[a]

| Lineage combination | Strain | | No. of independent trials | Inoculation level (%) | | Distribution (%) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | UVM I | | UVM II | |
| | A | B | | A | B | A | B | A | B |
| 2 vs. 1 | La22 | V518a | 5 | 47 | 53 | 64 | 36 | 100 | 0 |
| | | | | 44 | 56 | >99 | <1 | 100 | 0 |
| | | | | 50 | 50 | ND | ND | 100 | 0 |
| | | | | 48 | 52 | 100 | 0 | 97 | 3 |
| | | | | 46* | 54 | 87 | 13 | ND | ND |
| | | | | 46 | 54* | 95 | 5 | ND | ND |
| | La22 | 4542 | 1 | 53 | 47* | 89 | 11 | 100 | 0 |
| | | | | 53* | 47* | 95 | 5 | 100 | 0 |
| | La22 | 7418 | 1 | 39* | 61* | 56 | 44 | 100 | 0 |
| | | | | 39* | 61* | 64 | 36 | 83 | 17 |
| | V5a | 7418 | 1 | 55* | 45 | 55 | 45 | 81 | 19 |
| | | | | 55* | 45* | 38 | 62 | 93 | 7 |
| | V5a | V518a | 2 | 48 | 52 | 91 | 9 | 100 | 0 |
| | | | | 42 | 58 | 96 | 4 | 100 | 0 |
| | V5a | C1-109 | 2 | 54 | 46 | 100 | 0 | 100 | 0 |
| | | | | 46 | 54 | 100 | 0 | 100 | 0 |
| | V5a | 4542 | 1 | 53* | 47* | 80 | 20 | 98 | 2 |
| | | | | 53* | 47* | 75 | 25 | 100 | 0 |
| | C1-056 | C1-109 | 3 | 44 | 56 | ND | ND | 100 | 0 |
| | | | | 59 | 41 | 100 | 0 | 100 | 0 |
| | | | | 54 | 46 | 97 | 3 | 100 | 0 |
| | C1-056 | 4542 | 1 | 55* | 45 | 100 | 0 | 100 | 0 |
| | | | | 55* | 45* | 100 | 0 | 100 | 0 |
| | C1-056 | 7418 | 1 | 58* | 42* | 67 | 33 | 95 | 5 |
| | | | | 58* | 42* | 58 | 42 | 88 | 12 |
| | Hu4239 | 7418 | 1 | 42* | 58* | 60 | 40 | 93 | 7 |
| | | | | 42* | 58* | 50 | 50 | 85 | 15 |
| | Hu4239 | V518a | 2 | 47 | 53 | 48 | 52 | 96 | 4 |
| | | | | 44 | 56 | 91 | 9 | 90 | 10 |
| | Hu4239 | C1-109 | 2 | 52 | 48 | 96 | 4 | 100 | 0 |
| | | | | 45 | 55 | 100 | 0 | 100 | 0 |
| | Hu4239 | 4542 | 1 | 52* | 48* | 93 | 7 | 100 | 0 |
| | | | | 52* | 48* | 90 | 10 | 97 | 3 |
| 2 vs. 2 | La22 | V5a | 3 | 40 | 60 | 80 | 20 | 75 | 25 |
| | | | | 48 | 52 | 63 | 37 | 48 | 52 |
| | | | | 56 | 44 | 87 | 13 | ND | ND |
| | La22 | C1-056 | 2 | 52 | 48 | 76 | 24 | 100 | 0 |
| | | | | 49 | 51 | 88 | 12 | 91 | 9 |
| | La22 | Hu4239 | 4 | 47 | 53 | 100 | 0 | 100 | 0 |
| | | | | 52 | 48 | 73 | 27 | 92 | 8 |
| | | | | 60 | 40 | 100 | 0 | 86 | 14 |
| | | | | 53 | 47 | 51 | 49 | 80 | 20 |
| 1 vs. 1 | V518a | C1-109 | 2 | 49 | 51 | 76 | 24 | 100 | 0 |
| | | | | 56 | 44 | 85 | 15 | 97 | 3 |
| | V518a | 4542 | 1 | 51* | 49* | 45 | 55 | 45 | 55 |
| | | | | 51* | 49* | 40 | 60 | 31 | 69 |
| | 7418 | 4542 | 1 | 52* | 48* | 76 | 24 | 50 | 50 |
| | | | | 52* | 48* | 72 | 28 | 70 | 30 |
| | 7418 | V518a | 1 | 47* | 53* | 68 | 32 | 75 | 25 |
| | | | | 47* | 53* | 67 | 33 | 65 | 35 |

[a] See Table 3, footnotes *a* and *b*. ND, not done.

(Table 4), and 1,160 colonies (97%) were lineage 2 and only 40 colonies (3%) belonged to lineage 1 (data not shown). However, within lineage 1, the strains belonging to serotype 4b (strains C1-109, V518a, and 4542) seemed to be more affected by the lineage 2 competition than serotype 1/2b (strain 7418).

When two lineage 2 strains were coinoculated, both appeared in most cases after UVM I and UVM II selection, albeit in various proportions. A total of 576 colonies were tested from combinations between two lineage 2 strains (data not shown). Strain La22 (lineage 2) appeared to be a "strong" isolate and accounted for 80 to 100% of the colonies after growth in UVM I and UVM II with the other lineage 2 strains (Hu4239 and C1-056). When the four lineage 1 strains were tested against one another, only one combination (V518a against C1-109) resulted in complete dominance by one strain (V518a). This dominance could be explained by differences in

TABLE 5. Proportion of *Listeria monocytogenes* and *Listeria innocua* after coculturing in BHI broth at 30°C for 24 h

| Lineage | Strain | | No. of independent trials | Inoculation level (%)[a] | | Distribution in BHI[b] (%) | |
|---|---|---|---|---|---|---|---|
| | A | B | | A | B | A | B |
| 2 | La22 | R255a | 1 | 50 | 50 | 49 | 51 |
| | | | | 50 | 50 | 46 | 54 |
| 2 | C1-056 | R255a | 1 | 50 | 50 | 92 | 8 |
| | | | | 50 | 50 | 89 | 11 |
| 1 | V518a | R255a | 2 | 48 | 52 | 97 | 3 |
| | | | | 48 | 52 | 98 | 2 |
| | | | | 56 | 44 | 99 | 1 |
| | | | | 56 | 44 | 98 | 2 |
| 1 | C1-109 | R255a | 1 | 55 | 45 | 50 | 50 |
| | | | | 55 | 45 | 49 | 51 |
| 2 vs. 1 | La22 | V518a | 1 | 46 | 54 | 73 | 28 |
| | | | | 46 | 54 | 75 | 25 |

[a] Combinations inoculated in duplicate.
[b] Distribution was determined by performing plate counts on RLM.

generation time, 1.05 h (V518a) versus 1.31 h (C1-109) (Table 2).

***Listeria* versus *Listeria* in BHI.** To test if the selective pressure in UVM caused the difference in bias between lineage 1 and lineage 2 strains, a few combinations of *Listeria* strains were tested as cocultures in BHI (Table 5). A higher proportion of *L. monocytogenes* lineage 1 appeared when grown with *L. innocua* in BHI than when grown in UVM I and II. The proportions of lineage 2 strains were similar after growth in BHI as when grown in UVM I. When testing strain La22 (lineage 2) against strain V518a (lineage 1) in BHI, V518a appeared in higher proportions compared to the competition in UVM I. These results indicate that the selective compounds in UVM could be important for the bias during enrichment.

**Growth experiment with lineage 2 (La22) versus lineage 1 (V518a) in UVM I.** When grown as single cultures, strain La22 and strain V518a grew with identical growth rates in UVM I (Fig. 1A). To determine at what point during the selective enrichment the cultures interacted, samples were withdrawn every 4 h, and 80 colonies were isolated from each sampling point and subtyped to differentiate between the two strains. The growth rates of strains La22 and V518a were similar until the cell density reached approximately $10^6$ CFU/ml (Fig. 1B). After 24 h, the combination consisted of 91% La22 and 9% V518a, and after 30 h, no colonies of V518a could be isolated. Hence, interaction occurred between stain La22 and strain V518a only at high cell densities.

**Bioassay.** The interaction between strain La22 (lineage 2) and strain V518a (lineage 1) which was seen in the growth experiment together with the overall bias between lineage 1 and lineage 2 during enrichment in UVM indicate that members of lineage 2 strains and *L. innocua* could have produced inhibitory compounds. To test if such compounds were present, supernatants and cultures from both BHI and UVM I broth of all *Listeria* species were tested in a well diffusion assay against all *Listeria* species. No strains were inhibitory to any of the other *Listeria* strains in this assay (data not shown).

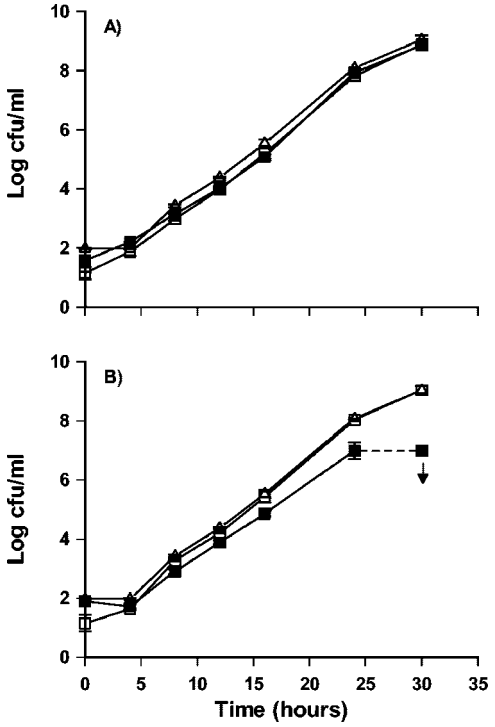


FIG. 1. A) Growth in UVM I of *Listeria monocytogenes* strain La 22 (□, lineage 2) and strain V518a (■, lineage 1) as single cultures andthe two strains grown in coculture. B) Growth in UVM I of *L. monocytogenes* strain La22 (□) and strain V518a (■) detected in the coculture by serotyping of 80 colonies and of a combination of the two strains (△).

## DISCUSSION

The *Listeria* selective procedures used during the enrichment of food and environmental samples are believed to allow detection of most (80 to 95%) samples containing *Listeria* species (31). However, the present study demonstrates that one of the common selective enrichment procedures (University of Vermont medium) used for the detection of *L. monocytogenes* in foods may not result in an equal representation of the subtypes of *L. monocytogenes* present in a food sample. Even though our data are based on a limited number of strains, a consistent pattern was found indicating that the method favors *L. monocytogenes* lineage 2 strains at the expense of lineage 1 strains. This bias is very unfortunate, since two of the three most common serotypes (4b and 1/2b) associated with human cases of listeriosis are lineage 1 strains (16, 20). Hence, epidemiological investigations where clinical strains, which are typically isolated by nonselective procedures, are compared to strains isolated from foods may have difficulties providing links between food sources and disease (12, 19). Several studies have noted that although serotype 4b is often involved in listeriosis, it is not found as commonly in foods or the environment as other serotypes (9, 16). Furthermore, Loncarevic et al. (17) found that direct plating yielded more *L. monocytogenes* clones than the enrichment procedure when sampling from foods. Our data indicate that this discrepancy could be caused by bias in the selection procedure used when sampling from food products and the environment.

Growth in nonselective medium was almost identical for the nine *Listeria* strains studied, and maximum cell densities of almost $10^{10}$ CFU/ml were reached. In agreement with this, Gracieux et al. (13) found that the growth of 40 *L. monocytogenes* strains in a nonselective medium (TSA) was similar. In contrast, the *Listeria* strains were differently affected by the selective agents present in UVM I, and growth of all strains was inhibited compared to their growth in BHI in agreement with other studies (5, 18).

Both *L. monocytogenes* and *L. innocua* are commonly isolated from and appear to grow equally well in foods (8, 14, 26, 29). Concern has been raised that *L. innocua* could outgrow *L. monocytogenes* during the detection enrichment procedures and hence mask the presence of the pathogen (6, 26). Some studies have indicated that *L. innocua* can grow faster in *Listeria* selective medium (18), whereas other studies (5) have not been able to demonstrate any differences in growth in selective medium. Our study clearly demonstrates that the presence of *L. innocua* may indeed mask the presence of *L. monocytogenes*; however, the selective advantage of *L. innocua* was only apparent when coinoculated with *L. monocytogenes* lineage 1 strains. *L. monocytogenes* lineage 2 strains grew as well as *L. innocua* strains during enrichment (Table 3). Most studies finding a dominance of *L. innocua* have used *L. monocytogenes* serotype 4b, which is a lineage 1 strain (6, 26).

The bias in strain selection when a lineage 1 and a lineage 2 *L. monocytogenes* strain were cocultured could not be explained by differences in growth rate in UVM I (Table 2). One may ask if a bias also occurs in food products in the presence of different *L. monocytogenes* strains. This seems not to be the case, since Porto et al. (27) inoculated five different *L. monocytogenes* strains (both lineage 1 and 2 strains) onto frankfurters, and all strains were detected after 28 days, although the percentages of lineage 2 strains were higher than those of lineage 1 strains.

The outcome postenrichment when two lineage 2 or two lineage 1 strains were cocultured could, in one case of lineage 2 (La22 versus C1-056) and one case of lineage 1 (V518a versus C1-109), be explained by significant differences in the individual growth rates of the strains ($P < 0.05$ and $P < 0.01$, respectively). In other cases (La22 versus Hu4239 or V5a), there were no significant differences in the individual growth rate yet the same strain dominated in all cases even though different percentage levels were found.

When comparing individual growth with growth in combination of strain La22 (lineage 2) and V518a (lineage 1) (Fig. 1), it appeared that La22 inhibited the growth of strain V518a in the coculture when a cell density of $10^6$ cell/ml was reached. When grown as monocultures, the growth of the two organisms was identical.

Changing the coculture experiment to the nonselective medium BHI shifted the bias for combinations between *L. monocytogenes* lineage 1 strains and *L. innocua*, but no shift was seen for combinations with *L. monocytogenes* lineage 2 strains and *L. innocua*. Additionally, strain V518a reached a higher percentage after growth in combination with strain La22 in BHI compared to the average level obtained after enrichment in UVM. Hence, the selective principles of UVM I and II (acriflavin and nalidixic acid) may contribute to the inhibition of one *Listeria* spp. strain by another during enrichment.

Nalidixic acid in UVM is used to suppress the growth of gram-negative bacteria and *Bacillus* spp. but has no effect on the growth of *L. monocytogenes* (15). Acriflavine is used in UVM to suppress non-*Listeria* gram-positive bacteria, but Jacobsen (15) found that *L. monocytogenes* strains were also inhibited by acriflavine and that different *L. monocytogenes* strains varied in their sensitivity to acriflavine. Acriflavine is used as the only supplement to suppress gram-positive bacteria in other *Listeria* selective enrichments medium such as BAMS, BCM, Fraser, and LRBS (32). Hence, the bias during enrichment in UVM of *L. monocytogenes* lineage 1 when present together with lineage 2 presented in this study is likely to occur when using other enrichment media.

Many gram-positive bacteria such as lactic acid bacteria produce bacteriocins that are inhibitory against *L. monocytogenes* (22, 30), and it has been shown that *L. innocua* can produce a bacteriocin(s) which inhibits *L. monocytogenes* (37). We did not, however, find that bacteriocin production (or other extracellular factors) caused the differential selection during growth in UVM I broth. Bacteriocin-negative lactic acid bacteria may be inhibitory to both lineage 1 and 2 *L. monocytogenes* strains, and it has been suggested that this is partly due to nutrient competition and that an interaction of this type could also take place between two *L. monocytogenes* strains (2, 24).

Our study indicates that the lineages of *L. monocytogenes* (28, 36), which are also reflected in the serotyping of the strains (21), could be physiologically different. Although large strain-to-strain variations are seen in all studies, some differences between lineages have been detected. Buncic et al. (3) found that serotype 1/2a (lineage 2) appeared to tolerate bacteriocins better than serotype 4b (lineage 1) and that lineage 2 strains appeared to attach better to surfaces than lineage 1 strains (1). Other studies have not been able to demonstrate a systematic, lineage-dependent difference between *L. monocytogenes* strains either in heat resistance (7) or in the ability to grow in sodium chloride (34). A shotgun DNA microarray was used to analyze 44 *L. monocytogenes* strains, and this revealed that 47 genes were absent in lineage 1 strains compared to lineage 2 strains (38). Some of the genes found in lineage 2 strains and not in lineage 1 strains are involved in stress response, transport of small molecules, or synthesis of cell wall molecules. One may speculate that while lineage 1 strains when growing as monocultures in UVM medium may cope with the stressful conditions and have a sufficient supply of nutrients such as amino acids, they cannot cope when cocultured with a more stress-tolerant lineage which is perhaps capable of more efficient uptake.

Listeriosis is a serious food-borne disease, and to implement appropriate control measures, one needs to understand the ecology of the virulent types as well as their niches, distribution, and transmission. To facilitate such investigations and assess the true distribution of serotypes and lineages, there is a need for the development of methods allowing all species and subtypes of *Listeria* to be detected.

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## REFERENCES


1. **Borucki, M. K., J. D. Peppin, D. White, F. Loge, and D. R. Call.** 2003. Variation in biofilm formation among strains of *Listeria monocytogenes*. Appl. Environ. Microbiol. **69:**7336–7342.
2. **Buchanan, R. L., and L. K. Bagi.** 1997. Microbial competition: Effect of culture conditions on the suppression of *Listeria monocytogenes* Scott A by *Carnobacterium piscicola*. J. Food Prot. **60:**254–261.
3. **Buncic, S., S. M. Avery, J. Rocourt, and M. Dimitrijevic.** 2001. Can food-related environmental factors induce different behaviour in two key serovars, 4b and 1/2a, of *Listeria monocytogenes*? Int. J. Food Microbiol. **65:**201–212.
4. **Capita, R., C. Alonso-Calleja, M. Prieto, M. Garcia-Fernandez, and B. Moreno.** 2001. Comparison of PALCAM and modified Oxford plating media for isolation of *Listeria* species in poultry meat following UVM II or Fraser secondary enrichment broths. Food Microbiol. **18:**555–563.
5. **Cornu, M., M. Kalmokoff, and J. P. Flandrois.** 2002. Modelling the competitive growth of *Listeria monocytogenes* and *Listeria innocua* in enrichment broths. Int. J. Food Microbiol. **73:**261–274.
6. **Curiale, M. S., and C. Lewus.** 1994. Detection of *Listeria monocytogenes* in samples containing *Listeria innocua*. J. Food Prot. **57:**1048–1051.
7. **De Jesus, A. J., and R. C. Whiting.** 2003. Thermal inactivation, growth, and survival studies of *Listeria monocytogenes* strains belonging to three distinct genotypic lineages. J. Food Prot. **66:**1611–1617.
8. **Duffy, G., D. Walsh, J. J. Sheridan, C. M. Logue, D. Harrington, I. S. Blair, and D. A. McDowell.** 2001. Comparison of selective and non-selective enrichment media in the detection of *Listeria monocytogenes* from meat containing *Listeria innocua*. J. Appl. Microbiol. **90:**994–999.
9. **Farber, J. M., and P. I. Peterkin.** 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiol. Rev. **55:**476–511.
10. **Fonnesbech Vogel, B., V. Fussing, B. Ojeniyi, L. Gram, and P. Ahrens.** 2004. High-resolution genotyping of *Listeria monocytogenes* by fluorescent amplified-fragment length polymorphism analysis as compared to typing by PFGE, RAPD, ribotyping and PCR-RFLP. J. Food Prot. **67:**1656–1665.
11. **Fonnesbech Vogel, B., H. H. Huss, B. Ojeniyi, P. Ahrens, and L. Gram.** 2001. Elucidation of *Listeria monocytogenes* contamination routes in cold-smoked salmon processing plants detected by DNA-based typing methods. Appl. Environ. Microbiol. **67:**2586–2595.
12. **Gilot, P., A. Genicot, and P. Andre.** 1996. Serotyping and esterase typing for analysis of *Listeria monocytogenes* populations recovered from foodstuffs and from human patients with listeriosis in Belgium. J. Clin. Microbiol. **34:**1007–1010.
13. **Gracieux, P., S. M. Roche, P. Pardon, and P. Velge.** 2003. Hypovirulent *Listeria monocytogenes* strains are less frequently recovered than virulent strains on PALCAM and Rapid′ L. mono media. Int. J. Food Microbiol. **83:**133–145.
14. **Gudbjörnsdttir, B., M.-L. Suihko, P. Gustavsson, G. Thorkelsson, S. Salo, A.-M. Sjöberg, O. Nichlasen, and S. Bredholt.** 2004. The incidence of *Listeria monocytogenes* in meat, poultry and seafood plants in the Nordic countries. Food Microbiol. **21:**217–225.
15. **Jacobsen, C. N.** 1999. The influence of commonly used selective agents on the growth of *Listeria monocytogenes*. Int. J. Food Microbiol. **50:**221–226.
16. **Jeffers, G. T., J. L. Bruce, P. L. McDonough, J. Scarlett, K. J. Boor, and M. Wiedmann.** 2001. Comparative genetic characterization of *Listeria monocytogenes* isolates from human and animal listeriosis cases. Microbiology **147:**1095–1104.
17. **Loncarevic, S., W. Tham, and M. L. Danielsson-Tham.** 1996. The clones of *Listeria monocytogenes* detected in food depend on the method used. Lett. Appl. Microbiol. **22:**381–384.
18. **Macdonald, F., and A. D. Sutherland.** 1994. Important differences between the generation times of *Listeria monocytogenes* and *Listeria innocua* in two *Listeria* enrichment broths. J. Dairy Res. **61:**433–436.
19. **Martinez, I., L. M. Rorvik, V. Brox, J. Lassen, M. Seppola, L. Gram, and B. Fonnesbech Vogel.** 2003. Genetic variability among isolates of *Listeria monocytogenes* from food products, clinical samples and processing environments, estimated by RAPD typing. Int. J. Food Microbiol. **84:**285–297.
20. **Mclauchlin, J.** 1990. Distribution of serovars of *Listeria monocytogenes* isolated from different categories of patients with listeriosis. Eur. J. Clin. Microbiol. **9:**210–213.
21. **Nadon, C. A., D. L. Woodward, C. Young, E. G. Rodgers, and M. Wiedmann.** 2001. Correlations between molecular subtyping and serotyping of *Listeria monocytogenes*. J. Clin. Microbiol. **39:**2704–2707.
22. **Nes, I. F., and H. Hole.** 2000. Class II antimicrobial peptides from lactic acid bacteria. Biopolymers **55:**50–61.
23. **Nilsson, L., L. Gram, and H. H. Huss.** 1999. Growth control of *Listeria monocytogenes* on cold-smoked salmon using a competitive lactic acid bacteria flora. J. Food Prot. **62:**336–342.
24. **Nilsson, L., Y. Y. Ng, J. N. Christiansen, B. L. Jorgensen, D. Grotinum, and L. Gram.** 2004. The contribution of bacteriocin to inhibition of *Listeria monocytogenes* by *Carnobacterium piscicola* strains in cold-smoked salmon systems. J. Appl. Microbiol. **96:**133–143.
25. **Patel, J. R., and L. R. Beuchat.** 1995. Evaluation of enrichment broths for their ability to recover heat-injured *Listeria monocytogenes*. J. Appl. Bacteriol. **78:**366–372.
26. **Petran, R. L., and K. M. J. Swanson.** 1993. Simultaneous growth of *Listeria monocytogenes* and *Listeria Innocua*. J. Food Prot. **56:**616–618.
27. **Porto, A. C. S., L. Wonderling, J. E. Call, and J. B. Luchansky.** 2003. Use of pulsed-field gel electrophoresis to monitor a five-strain mixture of *Listeria monocytogenes* in frankfurter packages. J. Food Prot. **66:**1465–1468.
28. **Rasmussen, O. F., P. Skouboe, L. Dons, L. Rossen, and J. E. Olsen.** 1995. *Listeria monocytogenes* exists in at least three evolutionary lines: evidence from flagellin, invasive associated protein and listeriolysin O genes. Microbiology **141:**2053–2061.
29. **Rawles, D. D., G. Flick, A. Diallo, and R. Croonenberghs.** 1995. Growth of mixed cultures of *Listeria monocytogenes* and *Listeria innocua* in blue-crab (*Callinectes-sapidus*) meat. J. Food Prot. **58:**1268–1270.
30. **Ross, R. P., S. Morgan, and C. Hill.** 2002. Preservation and fermentation: past, present and future. Int. J. Food Microbiol. **79:**3–16.
31. **Ryser, E. T., S. M. Arimi, M. M. C. Bunduki, and C. W. Donnelly.** 1996. Recovery of different *Listeria* ribotypes from naturally contaminated, raw refrigerated meat and poultry products with two primary enrichment media. Appl. Environ. Microbiol. **62:**1781–1787.
32. **Silk, T. M., T. M. T. Roth, and C. W. Donnelly.** 2002. Comparison of growth kinetics for healthy and heat-injured *Listeria monocytogenes* in eight enrichment broths. J. Food Prot. **65:**1333–1337.
33. **Suh, J. H., and S. J. Knabel.** 2001. Comparison of different enrichment broths and background flora for detection of heat-injured *Listeria monocytogenes* in whole milk. J. Food Prot. **64:**30–36.
34. **Vialette, M., A. Pinon, E. Chasseignaux, and M. Lange.** 2003. Growths kinetics comparison of clinical and seafood *Listeria monocytogenes* isolates in acid and osmotic environment. Int. J. Food Microbiol. **82:**121–131.
35. **Wiedmann, M.** 2002. Molecular subtyping methods for *Listeria monocytogenes*. J. AOAC Int. **85:**524–531.
36. **Wiedmann, M., J. L. Bruce, C. Keating, A. E. Johnson, P. L. McDonough, and C. A. Batt.** 1997. Ribotypes and virulence gene polymorphisms suggest three distinct *Listeria monocytogenes* lineages with differences in pathogenic potential. Infect. Immun. **65:**2707–2716.
37. **Yokoyama, E., S. Maruyama, and Y. Katsube.** 1998. Production of bacteriocin-like-substance by *Listeria innocua* against *Listeria monocytogenes*. Int. J. Food Microbiol. **40:**133–137.
38. **Zhang, C. M., M. Zhang, J. L. Ju, J. Nietfeldt, J. Wise, P. M. Terry, M. Olson, S. D. Kachman, M. Wiedmann, M. Samadpour, and A. K. Benson.** 2003. Genome diversification in phylogenetic lineages I and II of *Listeria monocytogenes*: Identification of segments unique to lineage II populations. J. Bacteriol. **185:**5573–5584.