The Distribution of *Listeria* in Pasture-Raised Broiler Farm Soils Is Potentially Related to University of Vermont Medium Enrichment Bias toward *Listeria innocua* over *Listeria monocytogenes*

**Aude Locatelli**, Micah A. Lewis and Michael J. Rothrock Jr.

1 Egg Safety and Quality Research Unit, U.S. National Poultry Research Center, Agricultural Research Service, United States Department of Agriculture, Athens, GA, United States, 2 Quality and Safety Assessment Research Unit, U.S. National Poultry Research Center, Agricultural Research Service, United States Department of Agriculture, Athens, GA, United States

The occurrence of *Listeria monocytogenes* has been widely investigated in the poultry production chain from the processing plant to the final product. However, limited data are available on *Listeria* species, including *Listeria monocytogenes*, in the poultry farm environment. Therefore, fecal and soil samples from 37 pastured poultry flocks from 10 all-natural farms over 3 years were assessed to determine the prevalence and diversity of *Listeria* within these alternative poultry farm environments using standard cultural and molecular methods. *Listeria* species were isolated in 15% of poultry farm samples and included *Listeria innocua* (65.7%), *L. monocytogenes* (17.4%), and *Listeria welshimeri* (15.1%). Additional multiplex PCR serotyping showed group 1/2a-3a to be the most dominant *L. monocytogenes* serovar group. Based on these results, monoculture growth experiments were conducted on four *Listeria* soil isolates (three *L. monocytogenes* isolates representing the three recovered serovar groups and one *L. innocua* isolate) to determine if culture medium [tripticase soy broth (TSB) and University of Vermont modified *Listeria* enrichment broth (UVM)], inoculum concentration (10² or 10⁵ CFU/ml), or incubation temperature (20, 30, and 42°C) differentially affected these *Listeria* species. Overall, very few significant growth differences were observed between the behavior of the three *L. monocytogenes* isolates (representing the three recovered serovar groups) under the growth conditions tested. Alternatively, at 30°C in UVM with the lower inoculum concentration, the *L. innocua* isolate had a significantly shorter lag phase than the *L. monocytogenes* isolates. In coculture growth studies under these same incubation conditions, the lag phase of *L. innocua* and *L. monocytogenes* was similar, but the final concentration of *L. innocua* was significantly higher than *L. monocytogenes*. However, cocultures in UVM for high inoculum concentration did not show preferential growth of *L. innocua* over *L. monocytogenes*. These results indicate that the use of UVM as an enrichment medium may preferentially allow *L. innocua* to outcompete *L. monocytogenes* at low concentrations, biasing the *Listeria* prevalence from these farm samples toward *L. innocua* and potentially underreporting the presence of *L. monocytogenes* in these environments.

**Keywords**: *Listeria monocytogenes*, *Listeria innocua*, pastured poultry, UVM enrichment medium, live production farms
INTRODUCTION

The genus *Listeria* is currently comprised of 17 species, including 11 *Listeria* species described since 2009 (1). However, the genus *Listeria sensu stricto* includes six species: *Listeria innocua*, *Listeria ivanovii*, *Listeria grayi*, *Listeria monocytogenes*, *Listeria seeligeri*, and *Listeria welshimeri*. These species are well documented and are known to be commonly found in different environments throughout the world (2–6). Among all the *Listeria* species, *L. monocytogenes* is recognized as one of the most important foodborne pathogens in many industrialized countries. This pathogen is responsible for listeriosis, a potentially fatal disease that may lead to abortion or serious cases of meningitis, encephalitis, and septicemia (7, 8). Although listeriosis infections are uncommon, mortality rates can reach 30% in at-risk population groups (9–11). In 2015 in the United States, *L. monocytogenes* was responsible for an estimated 116 cases of listeriosis, 111 hospitalizations, and 15 deaths (12).

*Listeria* species have been isolated from a wide variety of environments including nature (13) and urban areas (14), agricultural environments (15), food processing plants (16, 17), and retail food (18, 19). The occurrence of *Listeria* species is of special interest in the food production chain due to the significant threat that *L. monocytogenes* represents to public health (20, 21). Numerous studies have investigated the occurrence of *L. monocytogenes* in final products and in food-processing and retail environments, thought to be the main source of contamination for the final product (21, 22). However, limited information is available on *Listeria* prevalence in poultry farm production environments. In a farm-to-fork approach, it is necessary to assess the incidence of *L. monocytogenes* along the entire production chain and particularly at the primary production step (the farm environment), taking into account that it could be a potential source of this pathogen into food processing plants. Few studies have investigated and characterized *Listeria* species in the farm environment, with *L. innocua* being the predominant species found on grow-out farms, representing ≤78% of all isolated *Listeria* species (15, 23). Other species such as *L. ivanovii*, *L. monocytogenes*, *L. welshimeri*, and *L. seeligeri* have also been identified in environmental farm samples or chicken feces, but their detection remains infrequent (15, 23).

The initial isolation of *L. monocytogenes* may be difficult due to its low cell number within the larger indigenous microflora of environmental samples. Thus, the detection of *Listeria* species involves selective enrichment procedures. Numerous one-step and two-step enrichment broths have been described during the past 50 years (27), with the three most commonly used procedures being the (1) modified ISO 11290-1, (2) USDA-Food Safety Inspection Service (FSIS) Microbiology Laboratory Guide (MLG) method 8.10, and (3) U.S. Food and Drug Administration Bacteriological Analytical Method (FDA-BAM) method #10. Several studies have shown that the enrichment procedure can result in *L. monocytogenes* being overgrown by other non-pathogenic *Listeria* species in samples where multiple species are present (28–32). This has especially been demonstrated with *L. innocua*, whose presence may mask *L. monocytogenes* and lead to false negative results (33, 34). In addition, several studies have shown that among *Listeria* strains of food origins, *L. innocua* grows faster than *L. monocytogenes* in enrichment media cocoltures or food matrices (28–32). These observations raised the question whether the higher prevalence of *L. innocua* observed in samples from the farm environment is due to a differential growth of *Listeria* species during the enrichment process or reflect their true distribution in the environment.

While commercial, conventional production represents the majority of the U.S. poultry market, alternative production systems (e.g., organic, all-natural) are becoming more prevalent and there is very limited information related to the prevalence of *Listeria* spp. within this type of farm environment (35). Therefore, the goal of this work was twofold: (1) determine the prevalence and distribution of *Listeria* spp. within poultry-related environmental samples (feces and soil) during live production on pastured poultry farms and (2) evaluate whether the distribution of recovered *Listeria* spp. could be explained by a differential growth in the enrichment broth used in this study, or accurately reflected the native species distribution in the environment.

MATERIALS AND METHODS

Sample Collection

Ten farms within the southeastern United States were sampled over a period of 3 years (from 2014 to 2016), representing 37 pasture-raised broiler flocks. Farm descriptions are available in Table 1. Soil and feces samples were collected from the pasture where the flock was currently residing at the time of sampling. Samplings occurred three times during grow-out: (i) within a few days of being placed in the pasture, (ii) halfway through their time on pasture, and (iii) on the day the flock was processed. At each sampling time, the pasture area was divided into five separate sections, and five subsamples in each section were pooled into a single sample for each section (a total of five soil samples and five feces samples were collected on each sampling day). Soil samples were collected from the surface (0–7 cm) with sterile scoops, and feces samples were collected from fresh droppings on the soil surface. Gloves and scoops were changed between sample types and between sampling areas. Samples were transported back to the lab on ice and processed within 2 h of collection. A total of 1,110 samples (555 feces samples and 555 soil samples) were collected over the 3-year study period.

Culture-Based Detection and Isolation of *Listeria* Species from Soil and Feces Samples

Enrichment and isolation of *Listeria* from these environmental samples were performed using a modified version of the USDA-FSIS MLG 8.10 method (36). Three grams of fresh soil or feces were added to 9 ml of buffered peptone water (Acumedia, Lansing, MI, USA) in a filtered stomacher bag and were vigorously shaken for 30 s. As a pre-enrichment step, the stomached homogenates remained in the filtered stomacher bag and were incubated overnight at 35°C. This pre-enrichment step was followed by two enrichments in University of Vermont Modified
TABLE 1 | Characteristics of the 10 all-natural pastured poultry farms sampled over the 3-year period.

| Breed         | Farm A | Farm B | Farm C | Farm D | Farm E |
|---------------|--------|--------|--------|--------|--------|
| Flock size    | Freedom ranger | Freedom ranger | Cornish cross | Freedom ranger | Freedom ranger |
| No. of flocks | >500   | 10     | 1      | 4      | 4      |
| Length of grow-out (weeks) | 10–11 | 13     | 13     | 11     | 9      |
| Multiuse farm? | Yes | Yes | Yes | Yes | Yes |
| Animal types  | Layers, swine, beef cattle, and sheep | Layers, swine, horses, and goats | Layers, swine, horses, and goats | Layers, swine, beef cattle, and sheep | Layers, swine, beef cattle, and sheep |

Listeria Enrichment Broth (UVM; Remel, Lenexa, KS, USA) and Fraser Broth (Oxoid CM0895, Basingstoke, UK), both requiring overnight incubation for 24 h at 30°C. One loopful of the Fraser’s enrichment culture was streaked on Listeria selective agar (LSA, Oxoid CM0856, Basingstoke, UK) for the isolation of Listeria colonies. These plates were incubated overnight at 30°C, and on each plate three Listeria-like colonies per positive samples were picked and kept for further identification tests. Stock cultures were prepared by growing Listeria strains in tripticase soy broth (TSB; Acumedia, Lansing, MI, USA) at 37°C. After washing in sterile water, the cell pellet was suspended in a brain heart infusion (BHI) broth (Acumedia, Lansing, MI, USA) with 25% of glycerol, aliquoted (300 µl in microtubes) and frozen at −80°C until further utilization.

Characterization of Listeria Species and L. monocytogenes Serovar Groups by Multiplex PCR

The species of presumptive Listeria colonies recovered on LSA were determined by multiplex PCR (37). In short, speciation occurred using two multiplex PCR reactions, based on the size of PCR amplicons. Pool 1 contained the primers for the identification of L. ivanovii, L. grayi, and L. innocua, and pool 2 contained primers for the identification of L. welshimeri, L. monocytogenes, and L. seeligeri. A 25 µl PCR reaction was composed of 1× EconoTaq PLUS 2× Master Mix (Lucigen Corporation, Middleton, WI, USA), 1 µM of each livN, Igr, and lin2 reverse and forward primers (for Pool 1) or 1 µM of each lwe, Lmo, and le reverse and forward primers (for Pool 2) and quantity sufficient (qs) of water. For negative controls, sterile water was added instead of template DNA. The cycling program consisted of 1 cycle at 95°C for 9 min; 30 cycles at 94°C for 30 s, at 60°C for 30 s, and at 72°C for 1 min; and 1 cycle at 72°C for 7 min. The serovar group of isolates classified as L. monocytogenes was determined by multiplex PCR using five sets of primers (38). Briefly, one colony of L. monocytogenes isolates was thoroughly mixed in a 25 µl PCR reaction containing: 1× EconoTaq PLUS 2× Master Mix (Lucigen Corporation, Middleton, WI, USA), 1 µM of each Lmo0737, ORF2819, and ORF2210 reverse and forward primers, 1.5 µM of Lmo1118 reverse and forward primers, 0.2 µM of prs reverse and forward primers and qs water. For negative controls, sterile water was added instead of template DNA. PCR was performed with an initial denaturation step at 94°C for 3 min; 35 cycles of 94°C for 0.40 min, 53°C for 1.15 min and 72°C for 1.15 min; and 1 final cycle for 72°C for 7 min. PCR reactions were performed in an Eppendorf Mastercycler EP Gradient S (Eppendorf). After the completion of all cycles, 18 µl of PCR product was mixed with 3 µl of BlueJuice™ loading buffer (Invitrogen, Carlsbad, CA, USA) and separated on a 2% E-gel® with SYBR-safe™ (Invitrogen) along with 12 µl of E-Gel™ 1 kb Plus DNA Ladder (Invitrogen).

Bacterial Growth Experiments

Bacterial Strain Selection and Inoculum Preparation

Based on the two main Listeria species found from farm distribution data, three L. monocytogenes strains representing the different recovered serovar groups (1/2a-3a, 1/2b-3b-7, and 4b-4d-4c) and one L. innocua were selected for the growth experiments. Pre-cultures were prepared by inoculating 60 ml of TSB with 100 µl of the thawed stock culture and incubated for 24 h at 30°C while shaking (150 rpm). After 24 h, cell density was estimated spectrophotometrically by measuring the optical density (OD) at 600 nm (OD600nm) with the Thermo Scientific Spectronic 200™ (Fisher Scientific). Pre-cultures were initially diluted in TSB or UVM to a concentration of 10⁶ CFU/ml, and then serially diluted in TSB or UVM to obtain final inoculum concentrations of 10⁴ and 10⁵ CFU/ml.

Monoculture Growth Experiments of Listeria Strains

A volume of 0.4 ml of each culture (10⁴ and 10⁵ CFU/ml) for the four Listeria strains was aliquoted into wells of a
microplate (Honeycomb 2 cuvette plate; Labsystems, Inc., Franklin, MA, USA), with five repeats of each culture condition (strain × medium × concentration) per plate. Negative controls consisted on 0.4 ml of uninoculated TSB and UVM (five repeats) incubated along the cultures. For each culture, two independent plate repeats containing all treatment combinations were performed. The inoculated microplate was placed in a Bioscreen C microbiology reader (Thermo Electron Corp., West Palm Beach, FL, USA), which was operated by a computer with Growth Curves Software, v 2.28 (Transgalactic Ltd., Helsinki, Finland). The microbiology reader recorded the OD values of cultures at 20-min intervals after a plate shaking of 10 s at a medium speed (30 shakes/min). Three incubation temperatures were chosen, and the corresponding incubation times were adjusted to make sure that all growth curves reach the stationary phase by the end of the experiment. Plates were incubated at (i) 20°C, estimated soil temperature calculated upon the average of the atmospheric temperatures encountered during the sampling period (from March to August), for 48 h, (ii) 30°C, recommended temperature used for the enrichment procedure of Listeria spp. in UVM medium, for 24 h, and (iii) 42°C, expected temperature inside the chicken intestine, for 24 h.

Coculture Growth Experiments of L. monocytogenes Serovar Group 1/2a-3a and L. innocua

Since no significant growth differences were observed among the three L. monocytogenes isolates in the monoculture growth study, subsequent coculture growth studies with the L. innocua isolate were only performed with the L. monocytogenes 1/2a-3a isolate (the most prevalent serovar group found within the farm data). Three different coculture mixtures were used to observe coculture growth effects: (1) L. monocytogenes 1/2a-3a to L. innocua ratio of 10^2:10^5 CFU/ml in UVM, (2) L. monocytogenes 1/2a-3a to L. innocua ratio of 10^4:10^5 CFU/ml in UVM, and (3) L. monocytogenes 1/2a-3a to L. innocua ratio of 10^7:10^5 CFU/ml in TSB. As positive controls, monocultures of L. monocytogenes 1/2a-3a and L. innocua were tested under the same conditions (medium × concentration) as the coculture mixtures, while negative controls consisted of uninoculated TSB or UVM, respectively. Each coculture mixture was inoculated individually into a microplate along with positive and negative controls with a final inoculum volume of 0.4 ml for each condition. The OD was recorded at 30-min intervals after a brief plate shaking of 10 s at a medium speed (30 shakes/min) during the incubation at 30°C for 24 h. To quantify the growth of L. monocytogenes 1/2a-3a and L. innocua in coculture, 100 µl aliquots were sampled every hour from the microwell plate and serially 10-fold diluted. Appropriate dilutions were plated on Rapid L. mono medium (Bio-Rad, Hercules, CA, USA). Blue and white colonies were enumerated as L. monocytogenes 1/2a-3a and L. innocua, respectively.

Modeling the Microbial Growth Kinetics and Statistical Analysis

Growth curves were plotted based on OD values over time. Each bacterial growth curve was fitted to a modified Gompertz model using Matlab 2007b. The model equation is as follows:

\[ y = Ae^{-e^{\frac{\lambda \mu_{\text{max}}}{C}}}, \]

where \( y \) is the OD value measured, \( t \) is the time (h), \( \mu_{\text{max}} \) is the maximum specific growth rate (h^{-1}), \( A \) is the maximum OD value attained, and \( \lambda \) is the lag time (h). Within the m-file written in Matlab, the lsqcurvefit function (a nonlinear least-squares solver for data fitting) was utilized to fit the growth curves by first using the following Gompertz equation:

\[ y = Ae^{-e^{\frac{\lambda \mu_{\text{max}}}{C}}}. \]

Then, the A, B, and C terms were used to determine the growth parameters of interest in the model equation as follows:

\[ \mu_{\text{max}} = \frac{A-C}{e^{\lambda B-C}}, \]

\[ \lambda = \frac{B-1}{C}. \]

The raw data for each growth curve were graphed along with the resulting fit, and the R^2 value (coefficient of determination) for each resulting fit was calculated. A four-way analysis of variance (ANOVA) was performed separately on each growth parameter (\( \lambda \), \( \mu_{\text{max}} \), and A), followed by Tukey's post hoc test in R software v3.2.1. Factors included in the model were the Listeria strains, the culture medium, the inoculum concentration, and the incubation temperature. For the coculture experiments, \( \mu_{\text{max}} \) and stationary phase cell densities (equivalent to OD_{max}) were log10-transformed before ANOVA, and a Tukey's post hoc test was used to group treatments. For all analyses, differences among groups were considered significant if \( p \leq 0.05 \).

RESULTS AND DISCUSSION

Prevalence and Distribution of Listeria Species in Soil and Feces Collected from Pastured Poultry Farms

A total of 1,110 samples (555 feces samples and 555 soil samples) were collected from 37 flocks on 10 pastured poultry farms over a 3-year period, and the distribution of Listeria species varied according to the sampling year (Figure 1A), broiler farm (Figure 1B), and sample type (Figure 1C). Overall, Listeria species were detected on all the farms and isolated in 15% of samples (83 from feces and 85 from soils), which is in the range of Listeria species prevalences reported in poultry-related environmental samples (from 1.4 to 53%) such as broiler litter, farm feed, farm drinking water, soil, and grass (25, 26, 39, 40) as well as in poultry feces (4.7–17%) (23, 25). In our study, three species were isolated including L. innocua (65.7%), L. monocytogenes (17.4%), and L. welshimeri (15.1%), and each of these species were recovered from at least half of the broiler farms (80, 50, and 90%, respectively; Figure 1B). Although different Listeria species distributions were observed between farms, at least two Listeria species were recovered from all but one farm (Farm M), and all three species were recovered from soil samples in all 3 years of the study (Figure 1C). Listeria innocua has been
previously shown to be the predominant species isolated from the broiler farm environment (23–26, 40), while the detection of other non-pathogenic *Listeria* species, such as *L. welshimeri*, remains infrequent (23, 40), mostly because studies only focus on *L. monocytogenes* (41–43).

*L. monocytogenes* was isolated from 5.8, 0.3, and 1.0% of all samples collected in 2014, 2015, and 2016, respectively, with 87% (26/30) recovered during 2014 (Figure 1A) and 57% (17/30) of all *L. monocytogenes* isolates coming from the only flock sampled on Farm D in 2014 (Figure 1B). Overall, three *L. monocytogenes* serovar groups were identified: 1/2b-3b-7 (20%), and 4b-4d-4e (10%). Interestingly, over the 3-year sampling period, only one *L. monocytogenes*-positive flock (Farm I, 2014) harbored more than one serotype, demonstrating the potential clonal nature of *L. monocytogenes* within a flock or on a farm (44). The overall prevalence of *L. monocytogenes* on these 10 farms was low compared with other grow-out farm environments where 0–46.2% of the environmental and feces samples were *L. monocytogenes* positive (45), but the distribution of the serotypes was consistent with other studies that have characterized *L. monocytogenes* serotypes in broiler flocks (26, 41, 43). The prevalence of *L. monocytogenes* contamination may be dependent on the type of production system. A significant difference between caged- and floor-reared hens was observed with a greater detection of *L. monocytogenes* in dust samples from floor-reared hens in *L. monocytogenes*-positive flocks (41). In alternative systems, broilers are raised in less controlled environments than conventional systems and are more likely to be in contact with *L. monocytogenes* known to be widely spread in soil and vegetation (35).

Poultry farms frequently have other animals (beef cattle, sheep, goats, or swine) and pets present on the production site (35). These animals can be reservoirs for and play a role in the proliferation and deposition of *L. monocytogenes* into the environment. In our study, all but two farms had other animals raised in close proximity to the broiler flocks during the sampling period, but we did not investigate the possible genotype matching between animal species. Generally, the presence of other animals on the farm increase the risk factor associated with pathogenic bacteria contamination of poultry flocks (42, 46). This has been shown with *Campylobacter* spp. where adjacent broiler flocks and cattle appear to be the most frequently identified animals with broiler-flock matching *Campylobacter* spp. isolates (47). Another study has reported an increased risk of *L. monocytogenes* contamination in laying hen flocks when pets were present on the production site (42).

### Monoculture Growth Experiments of *L. innocua* and *L. monocytogenes* Isolated from Pastured Poultry Farm Soils

**Growth Curve Modeling and Determination of Bacterial Growth Parameters**

Our field results showed a higher prevalence of *L. innocua* compared with *L. monocytogenes* on pastured poultry grow-out farms, which has been supported by other studies reporting the incidence and characterization of *Listeria* species in the commercial poultry farm environment (15, 23, 24, 26). In terms of food safety interests, there is a question as to whether there is any physiological basis for the dominance of *L. innocua* over *L. monocytogenes* within the poultry farm environment, and whether this dominance related to preferential growth. To determine if this environmental dominance of *L. innocua* over *L. monocytogenes* may be linked to growth conditions (e.g., initial concentration, growth temperature, and growth medium), monoculture and coculture growth studies were performed. Three *L. monocytogenes* isolates (one strain of each serovar groups: 1/2a-3a, 1/2b-3b-7, and 4b-4d-4e) and one *L. innocua*
isolate were selected to compare their growth capacity in liquid media. Bacterial growth was monitored by recording the OD of a culture in growth media (TSB and UVM) inoculated at different initial concentrations (10^2 and 10^5 CFU/ml) and incubated at 20°C (average environmental temperature), 30°C (UVM enrichment temperature according to USDA-FSIS MLG 8.10), or 42°C (broiler body temperature). Curve modeling was performed with the Gompertz function that fits the data with R^2 values ranging from 0.674 to 0.998, indicating a good fit. From the modified Gompertz equation, three relevant parameters [lag time (λ), maximum specific growth rate (μ_{max}), and maximum OD (OD_{max})] were determined for each curve and subsequently used to statistically compare the bacterial growth of the Listeria strains under the different cultural conditions. Using a four-way ANOVA (Tables S1–S3 in Supplementary Material for λ, μ_{max}, and OD_{max}, respectively), we investigated whether the culture medium, the inoculum concentration, and the incubation temperature could explain the global variation observed between the growth curves. In the same model, we more specifically examine the growth differences between the four Listeria isolates for a single culture condition. The parameters λ, μ_{max}, and OD_{max} representing bacterial growth characteristics were used in the model.

Effect of Culture Medium, Inoculum Concentration, and Incubation Temperature on Lag Time (λ)

Unsurprisingly, λ was significantly shorter for the higher inoculum concentrations for all strains, enrichment media, and incubation temperatures (F = 801, p < 0.0001; Figure 2). This is in agreement with other studies that have evidenced the importance of inoculum concentration on the ability of a microbial population to initiate growth (48, 49). While Baranyi et al. showed that as the cell numbers in the inoculum decrease, λ increases (50, 51), other studies have reported an effect of the inoculum size only under stressful conditions (49, 52). Increasing incubation temperature significantly decreased λ (F = 174, p < 0.0001) in both TSB and UVM enrichment media for all Listeria strains, as has been showed in other growth media for both L. monocytogenes and L. innocua (28, 49, 52, 53). The temperature-dependent effect was significantly greater in the low initial concentration treatments compared with the higher initial inoculum treatments (F = 77, p < 0.0001). While lag time was significantly shorter in TSB compared with UVM, this was the weakest association of the major growth variables tested (F = 60, p < 0.05).

Effect of Culture Medium, Inoculum Concentration, and Incubation Temperature on Maximum Specific Growth Rate (μ_{max})

While many of the growth variables tested significantly effected μ_{max} by far the strongest association was to the enrichment medium (F = 2431, p < 0.0001), where the four Listeria strains grew faster in TSB than UVM (Figure 3). This result is in agreement with the general trend of Listeria strains from food origin showing a faster growth in general growth media (e.g., BHI, TSB + yeast extract) than Listeria enrichment media (UVM, Fraser, and Half-Fraser) (30, 33). We also observed that Listeria strains grew significantly faster at lower incubation temperatures, peaking at 30°C, and this effect was amplified in TSB medium (F = 81, p < 0.0001). This is in agreement with Duh and Schaffner (28), who showed that Listeria strains grew faster at temperatures below 41°C in general growth media (28). The growth variable with the weakest significant association to μ_{max} was initial inoculum concentration, where its effect were only observed in the 42°C treatments (F = 25, p > 0.0001).

Effect of Culture Medium, Inoculum Concentration, and Incubation Temperature on OD_{max}

As was observed for μ_{max}, the enrichment medium was the dominant growth variable affecting OD_{max} (F = 1481, p < 0.0001) with significantly higher maximum optical densities found in the treatments grown in TSB (Figure 4). This is consistent with other data reporting a higher final cell density in the non-selective culture medium BHI than in selective enrichment media.
UVM or Half-Fraser (34, 54). For all *Listeria* strains, the OD$_{\text{max}}$ significantly increased with decreasing incubation temperatures, especially in the UVM treatments ($F = 193$, $p < 0.0001$). Unlike $\lambda$ or $\mu_{\text{max}}$, initial inoculum concentrations did not have a significant effect of OD$_{\text{max}}$ overall ($F = 0.01$, $p > 0.05$), although limited effects were observed at treatments incubated at 42°C.

**Comparing Monoculture Growth between *L. monocytogenes* and *L. innocua* Strains**

While the general effects of the above variables on growth of *Listeria* spp. overall, the question of the differential effect between specific *Listeria* species still remained. While there were some exceptions, generally there were no significant differences between the three *L. monocytogenes* strains in terms of $\lambda$, $\mu_{\text{max}}$, or OD$_{\text{max}}$ and regardless of enrichment media, the *L. innocua* strain was unable to grow at broiler body temperature (42°C) when the initial inoculum concentrations was $10^2$ CFU/ml (TSB$\text{Low}$ and UVM$\text{Low}$). Significant differences in lag time between the three *L. monocytogenes* strains and the *L. innocua* strain varied based on the growth variables (Figure 2). At the average environmental and UVM enrichment temperatures (20 and 30°C, respectively), the lag time of *L. innocua* was significantly shorter than the *L. monocytogenes* strains in UVM, especially for low inoculum concentrations ($p < 0.05$; Figures 2A, B, respectively). However, at broiler body temperatures (42°C), *L. innocua* only grew in the high initial inoculum treatments (TSB$\text{High}$ and UVM$\text{High}$), where there were no significant differences between the four *Listeria* strains (Figure 2C). No significant differences in $\lambda$ between *L. innocua* and *L. monocytogenes* were found in any of the TSB treatments. Previous studies comparing the growth of *L. monocytogenes* and *L. innocua* strains mostly from food origins.
have reported shorter $\lambda$ for $L.\ innocua$ in Fraser (incubated at 30°C), and Half-Fraser (incubated at 37°C) enrichment media (31) and at lower incubation temperatures ($\leq 8^\circ C$) (28).

While significant differences in $\mu_{max}$ were observed between the four Listeria strains used in this study, there were no consistent trends based on initial inoculum concentration, incubation temperature or enrichment medium (Figure 3). Only in two treatment combinations were there species-specific significant differences in $\mu_{max}$ with $L.\ monocytogenes$ growing faster than $L.\ innocua$ in TSBHigh at 30°C (Figure 3B) and $L.\ innocua$ growing faster than $L.\ monocytogenes$ at 42°C in the UVMHigh treatment (Figure 3C). In contrast to previously reported findings, there were no significant differences found in $\mu_{max}$ between the $L.\ monocytogenes$ and $L.\ innocua$ isolates in UVM at 30°C, conditions used for the Listeria enrichment process (28, 31, 33). However, the studies comparing the generation time or the growth rate have shown a faster growth of $L.\ innocua$ compared with $L.\ monocytogenes$ at temperatures below 40°C only in certain culture media (28, 31, 33), which may explain the similar $\mu_{max}$ between $L.\ innocua$ and $L.\ monocytogenes$ in our study. In addition, a high level of heterogeneity in growth behavior within $L.\ innocua$ and $L.\ monocytogenes$ strains can lead to equivalent $\mu_{max}$ between the slowest $L.\ innocua$ and the fastest $L.\ monocytogenes$ (30, 55).

There were very few strain-specific differences in the maximum OD (OD$_{max}$) for any of the growth variables, with the significant differences found at 42°C (Figure 4C). Among those differences, only under one treatment condition (TSBHigh) were there significant differences between $L.\ monocytogenes$ and $L.\ innocua$, so overall the maximum cell density in culture was unaffected by the Listeria species. No differences were observed between the OD$_{max}$ of $L.\ innocua$ and $L.\ monocytogenes$ species in UVM at 30°C as reported in studies using Half-Fraser and Fraser (30, 31). However, these results are highly dependent on the experiment and opposite trends are also reported showing an higher final population density of $L.\ innocua$ than $L.\ monocytogenes$ in enrichment media (29, 54).

Differential Growth of $L.\ monocytogenes$ 1/2a-3a and $L.\ innocua$ in Coculture Growth Experiments

Using the cultural conditions for the initial enrichment step for the USDA-FSIS MLG 8.10 $L.\ monocytogenes$ enrichment method (UVM, 30°C) we found that $L.\ innocua$ exhibited a significantly shorter lag time than the $L.\ monocytogenes$ strains in monocultures, especially for low initial inoculum concentrations ($10^2$ CFU/ml). To determine if $L.\ innocua$ has any direct competitive growth advantages over $L.\ monocytogenes$ in UVM, coculture experiments were performed using the $L.\ innocua$ strain and the $L.\ monocytogenes$ 1/2a-3a strain (the most prevalent serovar group from the farm surveys). When both strains were inoculated into the coculture at $10^3$ CFU/ml (Figure 5A), there were no significant differences in $\lambda$, $\mu_{max}$ or stationary phase cell density (similar to OD$_{max}$), although $L.\ monocytogenes$ densities did begin to exceed $L.\ innocua$ cell densities after 24 h. When both strains started at the lower inoculum level ($10^2$ CFU/ml), while $\lambda$ and $\mu_{max}$ were similar, $L.\ innocua$ reached a significantly higher stationary phase cell density compared with $L.\ monocytogenes$ ($F = 31, p < 0.01$; Figure 5B). Conversely, when the cocultures inoculated at the lower initial concentrations were grown in TSB, $L.\ monocytogenes$ demonstrated significantly higher stationary phase cell densities compared with $L.\ innocua$ ($F = 19, p < 0.05$), with $L.\ monocytogenes$ cell densities being ~3x greater than $L.\ innocua$ (Figure 5C). When comparing the growth curve parameters among the three coculture experiments, only the stationary phase cell density was significantly affected at the species-level (Table 2).

Unlike the monoculture results using the UVM protocol from the USDA-FSIS MLG 8.10 method, no significant lag time
that this advantage comes more from a disadvantage of it appears that (28, 31, 33). L. innocua L. monocytogenes and shown that enrichment/culture media can differentially effect L. innocua advantage that possesses, and previous studies have growing under these conditions, rather than a specific cytogenes ing significantly higher densities of L. innocua compared with L. monocytogenes two enrichments in the USDA-FSIS MLG 8.10 protocol, hav- ing Listeria could potentially or partially explain the prevalence of L. innocua production farms and processing plants, this enrichment bias responsible for the testing of foodborne pathogens from broiler within the USDA-FSIS MLG 8.10 method, and USDA-FSIS is and lating L. monocytogenes and L. innocua spp. found on poultry farms (15, 23–26).

In our study, we found that L. innocua is more prevalent than the foodborne pathogen L. monocytogenes in soil and feces samples collected from pastured poultry farms, which is consistent with conventional poultry farms. Mono- and coculture growth experiments showed that under cultural conditions used in the first enrichment step of the USDA-FSIS MLG 8.10 L. monocytogenes method (UVM, 30°C), L. innocua had a significantly shorter lag phase (monoculture) and a significantly higher stationary phase cell density (coculture) compared with L. monocytogenes; these growth advantages occurred at low initial inoculum concentrations simulating the low levels of Listeria species encountered in the environment. Based on these results, it is possible that UVM enrichment medium either preferentially supports L. innocua growth over L. monocytogenes, or preferentially restricts L. monocytogenes growth, and that this enrichment step may be biasing the recovery of L. innocua over L. monocytogenes from live production samples. Considering the public health importance of accurately identifying the source of L. monocytogenes outbreaks, future work will need to understand the cultural and molecular mechanisms of this preferential L. innocua growth in UVM, and alternative enrichment methods for L. monocytogenes may need to be considered.

**AUTHOR CONTRIBUTIONS**

AL and MR helped to develop experiments, analyze data, and prepare manuscript. ML helped in data analysis and manuscript preparation.

**ACKNOWLEDGMENTS**

The authors would like to acknowledge Laura Lee Rutherford and Cheryl Pearson Gresham for their assistance in sample acquisition and processing as well as Tori McIntosh for the molecular analyses of Listeria isolates. They would also like to thank Dr. Arthur Hinton for access his equipment and Kimberly Ingram for training on the microplate growth reader equipment and software.

**FUNDING**

This work was funded under USDA ARS CRIS # 6040-32000-011-00-D entitled “Reduction of Invasive Salmonella enterica in Poultry through Genomics, Phenomics, and Field Investigations of Small Multi–Species Farm Environments.”

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fvets.2017.00227/full#supplementary-material.
REFERENCES

1. Orsi RH, Wiedmann M. Characteristics and distribution of Listeria spp., including Listeria species newly described since 2009. *Appl Microbiol Biotechnol* (2016) 100:5273–87. doi:10.1007/s00253-016-7352-2

2. Hofer E, Ribiero R, Pintoosa DP. Species and serovars of the genus Listeria isolated from different sources in Brazil from 1971 to 1997. *Mem Inst Oswaldo Cruz* (2000) 95:615–20. doi:10.1590/S0074-08662000000500005

3. Chapin TK, Nightingale KK, Worobo RW, Wiedmann M, Strawn LK. Geographical and meteorological factors associated with isolation of Listeria species in New York State produce and natural environments. *J Food Prot* (2014) 77:1919–28. doi:10.4315/0362-028X.JFP-14-132

4. Linke K, Rückerl I, Brugger K, Karpiskova R, Walland J, Muri-Klinger S, et al. Reservoirs of Listeria species in three environmental ecosystems. *Appl Environ Microbiol* (2014) 80:5838–92. doi:10.1128/AEM.01018-14

5. Mcauley CM, Mcmillan K, Moore SC, Fegan N, Fox EM. Prevalence and characterization of foodborne pathogens from Australian dairy farm environments. *J Dairy Sci* (2014) 97:7402–12. doi:10.3168/jds.2014-8735

6. Stea EC, Purdue LM, Jamieson RC, Yost CK, Hansen LT. Comparison of the prevalences and diversities of Listeria species and Listeria monocytogenes in an urban and a rural agricultural watershed. *Appl Environ Microbiol* (2015) 81:3812–22. doi:10.1128/AEM.00416-15

7. Farber J, Peterkin P. Listeria monocytogenes, a food-borne pathogen. *Microbiol Rev* (1991) 55:476–511.

8. Low J, Donachie W. A review of Listeria monocytogenes and listeriosis. * Vet J* (2014) 198:53–9. doi:10.1016/j.tvjl.2013.08.006

9. Siegman-Igra Y, Levin R, Weinberger M, Golan Y, Schwartz D, Samra Z, et al. Diversity of Listeria monocytogenes infection in Israel and review of cases worldwide. *Emerg Infect Dis* (2002) 8:305–10. doi:10.3201/eid0803.010195

10. Wing EJ, Gregory SH. Listeria monocytogenes: clinical and experimental update. *J Infect Dis* (2002) 185:318–24. doi:10.1086/338465

11. Painter J, Slutsker L. Listeriosis in humans. In: Ryser ET, Marth EH, editors. *Listeria, Listeriosis and Food Safety – Third Edition*. Boca Raton, FL: CRC Press (2007). p. 75–95.

12. CDC. Foodborne Diseases Active Surveillance Network (FoodNet): FoodNet 2015 Surveillance Report (Final Data). Atlanta, GA: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention (2017).

13. Saunders BD, Wiedmann M. Ecology of Listeria species and L. monocytogenes in the natural environment. In: Ryser ET, Marth EH, editors. *Listeria, Listeriosis and Food Safety – Third Edition*. Boca Raton, FL: CRC Press (2007). p. 21–53.

14. Saunders BD, Overdevest J, Fortes E, Windham K, Schukken Y, Lembo A, et al. Diversity of Listeria species in urban and natural environments. *Appl Environ Microbiol* (2012) 78(12):4420–33. doi:10.1128/AEM.00822-12

15. Dahshan H, Merwad A, Mohamed TS, Listeria species in broiler poultry farms: potential public health hazards. *J Microbiol Biotechnol* (2016) 26:1551–6. doi:10.4103/0972-028X.160005

16. Martin B, Perich A, Gómez D, Yanguela J, Rodríguez A, Garriga M, et al. Listeria monocytogenes in intestinal contents of healthy animals in Japan. *J Vet Med Sci* (1991) 53:873–5. doi:10.1292/jvms.53.873

17. Sasaki Y, Haruna M, Murakami M, Hayashida M, Takahashi N, Urushiyama T, et al. Listeria monocytogenes in ready-to-eat products in retail and food service environments. *J Appl Bacteriol* (1996) 81:641–50. doi:10.1111/j.1365-2672.1996.tb01966.x

18. Petersen L, Madsen M. Listeria spp. in broiler flocks: recovery rates and species distribution investigated by conventional culture and the EiaFoss method. *Int J Food Microbiol* (2000) 58:113–6. doi:10.1016/S0168-1605(00)00258-0

19. Milillo S, Stout J, Hanning I, Clement A, Fortes E, Den Bakker H, et al. Listeria monocytogenes and hemolytic Listeria innocua in poultry. *Poult Sci* (2012) 91:2158–63. doi:10.3382/ps.2012-02292

20. Donnelly CW, Nyachuga DG. Conventional methods to detect and isolate Listeria monocytogenes. In: Ryser ET, Marth EH, editors. *Listeria, Listeriosis and Food Safety – Third Edition*. Boca Raton, FL: CRC Press (2007). 215 p.

21. Duh Y-H, Schaffner DW. Modeling the effect of temperature on the growth rate and lag time of Listeria innocua and Listeria monocytogenes. *J Food Prot* (1993) 56:205–10. doi:10.3382/ps.2003-028X-56.3.205

22. Peter T, Kanzaki M, Maruyama T, Inoue S, Kaneuchi C. Prevalence of Listeria monocytogenes species newly described since 2009. *Appl Microbiol* (2010) 42:3819–22. doi:10.1128/JCM.00258-00

23. Carvalheira A, Eusébio C, Silva J, Gibbs P, Teixeira P. Influence of Listeria innocua on the growth of Listeria monocytogenes. *Food Control* (2010) 21:1492–6. doi:10.1016/j.foodcont.2010.04.021

24. Curiale MS, Lewus C. Detection of Listeria monocytogenes in samples containing Listeria innocua. *J Food Prot* (1994) 57:1048–51. doi:10.3163/0362-028X-57.12.1048

25. Oracová K, Trnčíková T, Kuchtová J, Kaciková E. Limitation in the detection of Listeria monocytogenes in food in the presence of competing Listeria innocua. *J Appl Microbiol* (2008) 104:429–37. doi:10.1111/j.1365-2672.2007.0354.x

26. Rozema JL, Hiitt KL, Guard JJ, Jackson CR. Antibiotic resistance patterns of major zoonotic pathogens from all-natural, antibiotic-free, pasture-raised broiler flocks in the southeastern United States. *J Environ Qual* (2016) 45:593–603. doi:10.2134/jeq2015.07.0366

27. USDA-FSIS. *Isolation and Identification of Listeria monocytogenes from Red Meat, Poultry, Ready-to-Eat Sillariformes (Fish) and Egg Products, and Environmental Samples*. (2017). Availble from: https://www.fsis.usda.gov/wcm/content/1710feb8-7e69-4e6c-92fc-fd290fbf92/MLG-8.pdf?MOD=AJPERES

28. Huang B, Eglezos S, Heron BA, Smith H, Graham T, Bates J, et al. Comparison of multiplex PCR with conventional biochemical methods for the identification of Listeria spp. isolates from food and clinical samples in Queensland, Australia. *J Food Prot* (2007) 70:1874–80. doi:10.3163/0362-028X-70.8.1874

29. Doumith M, Buchrieser C, Glaser P, Jacquet C, Martin P. Differentiation of the major Listeria monocytogenes serovars by multiplex PCR. *J Clin Microbiol* (2004) 42:3819–22. doi:10.1128/JCM.42.8.3819-3822.2004

30. Jones D, Anderson K, Guard J. Prevalence of coliforms, Salmonella, Listeria, and Campylobacter associated with eggs and the environment of conventional cage and free-range egg production. *Poult Sci* (2012) 91:1195–202. doi:10.3382/ps.2011-01795

31. Dham A, Verma AK, Rajagunalan S, Kumar A, Tiwari R, Chakraborty S, et al. *Listeria monocytogenes* infection in poultry and its public health importance.
Differential Media-Based Listeria Growth

with special reference to food borne zoonoses. Pak J Biol Sci (2013) 16:301–8. doi:10.3923/pjbs.2013.301.308

41. Chemaly M, Toquin M-T, Le Nôtre Y, Fravalo P. Prevalence of Listeria monocytogenes in poultry production in France. J Food Prot (2008) 71:1996–2000. doi:10.4315/0362-028X.71.10.1996

42. Aury K, Le Bouquin S, Toquin M-T, Huneau-Salaün A, Le Nôtre Y, Allain V, et al. Risk factors for Listeria monocytogenes contamination in French laying hens and broiler flocks. Prev Vet Med (2011) 98:271–8. doi:10.1016/j.prevetmed.2010.11.017

43. Aury-Hainry K, Le Bouquin S, Labbé A, Petetin I, Chemaly M. Listeria monocytogenes contamination in French breeding and fattening turkey flocks. J Food Prot (2011) 74:1096–103. doi:10.4315/0362-028X.JFP-10-540

44. Haase JK, Didelot X, Lecuit M, Korkeala H, Achtman M. The ubiquitous nature of Listeria monocytogenes clones: a large-scale Multilocus Sequence Typing study. Environ Microbiol (2014) 16:405–16. doi:10.1111/1462-2920.12342

45. Rothrock MJ, Davis ML, Locatelli A, Bodie A, McIntosh TG, Donaldson JR, et al. Listeria occurrence in poultry flocks: detection and potential implications. Front Vet Sci (2017) 4:125. doi:10.3389/fvets.2017.00125

46. Torralbo A, Borge C, Allepuz A, Garcia-Bocanegra I, Sheppard SK, Perea A, et al. Prevalence and risk factors of Campylobacter infection in broiler flocks from southern Spain. Prev Vet Med (2014) 114:106–13. doi:10.1016/j.prevetmed.2014.01.019

47. Agunos A, Waddell L, Leger D, Taboada E. A systematic review characterizing on-farm sources of Campylobacter spp. for broiler chickens. PloS One (2014) 9:e104905. doi:10.1371/journal.pone.0104905

48. Pastucal C, Robinson T, Ocio M, Aboaia O, Mackey B. The effect of inoculum size and sublethal injury on the ability of Listeria monocytogenes to initiate growth under suboptimal conditions. Lett Appl Microbiol (2001) 33:357–61. doi:10.1046/j.1472-765X.2001.01012.x

49. Robinson TP, Aboaia OO, Kaloti A, Ocio MJ, Baranyi J, Mackey BM. The effect of inoculum size on the lag phase of Listeria monocytogenes. Int J Food Microbiol (2001) 70:163–73. doi:10.1016/S0168-1605(01)00541-4

50. Baranyi J. Comparison of stochastic and deterministic concepts of bacterial lag. J Theor Biol (1998) 192:403–8. doi:10.1006/jtbi.1998.0673

51. Baranyi J, Pin C. Estimating bacterial growth parameters by means of detection times. Appl Environ Microbiol (1999) 65:732–6.

52. Koutsoumanis KP, Sofos JN. Effect of inoculum size on the combined temperature, pH and aw limits for growth of Listeria monocytogenes. Int J Food Microbiol (2005) 104:83–91. doi:10.1016/j.ijfoodmicro.2005.01.010

53. Francois K, Devlieghere F, Smet K, Standaert A, Geeraerd A, Van Impe J, et al. Modelling the individual cell lag phase: effect of temperature and pH on the individual cell lag distribution of Listeria monocytogenes. Int J Food Microbiol (2005) 100:41–53. doi:10.1016/j.ijfoodmicro.2004.10.032

54. Bruhn JB, Vogel BF, Gram L. Bias in the Listeria monocytogenes enrichment procedure: lineage 2 strains outcompete lineage 1 strains in University of Vermont selective enrichments. Appl Environ Microbiol (2005) 71:961–7. doi:10.1128/AEM.71.2.961-967.2005

55. MacDonald F, Sutherland AD. Important differences between the generation times of Listeria monocytogenes and List. innocua in two Listeria enrichment broths. J Dairy Res (1994) 61:433–6. doi:10.1017/S0022029900030879

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer KG declared a shared affiliation, with no collaboration, with the authors to the handling editor.

Copyright © 2017 Locatelli, Lewis and Rothrock. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.