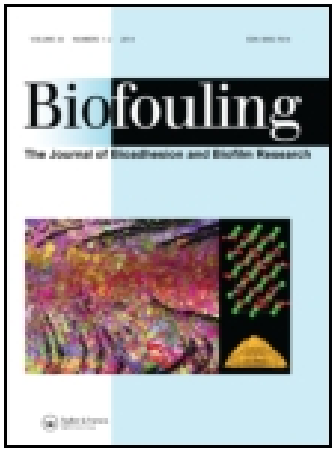

Biofouling: The Journal of Bioadhesion and Biofilm Research

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gbif20

Multivariate analysis reveals differences in biofilm formation capacity among Listeria monocytogenes lineages

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Published online: 14 Nov 2014.

Biofouling, 2014
Vol. 30, No. 10, 1199–1209, http://dx.doi.org/10.1080/08927014.2014.980818

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(*Received 29 August 2014; accepted 21 October 2014*)

Biofilm formation capacity evaluated under identical conditions differs among *Listeria monocytogenes* lineages. The approach of using one set of factors or one variable at a time fails to explain why some lineages are more prevalent than others in certain environments. This study proposes the use of multivariate analysis to compare biofilm formation by various strains and describes the ecological niches of *L. monocytogenes* lineages. Nutrient availability, temperature, pH and water activity (a_w) at three different levels were used to determine biofilm formation by 41 strains. Despite the high degree of similarity (≤ 80%), distinct lineage-associated biofilm formation patterns were identified. A linear regression model for each strain and a principal component analysis of regression coefficients indicated that Lineages I and III have different, but overlapping, ecological niches. This study is the first to report the use of multivariate analyses to compare biofilm formation by various isolates of *L. monocytogenes*.

Keywords: ecology; environment; pH; temperature; nutrient; biofilm

Introduction

Researchers consider *L. monocytogenes* to be ubiquitous because it is widespread in natural and human-made environments. Surface natural waters (Lyautey et al. 2007), as well as farms (Fenlon 1986), food processing facilities (Barbalho et al. 2005; Chao et al. 2006; Guerini et al. 2007) and households (Beumer et al. 1996; Sergelidis et al. 1997; Doorn et al. 2007) are environments that may serve as contamination routes or reservoirs of the pathogen (Oliver et al. 2007).

From a phylogenetic point of view, *L. monocytogenes* has been divided into four evolutionary lineages: I, II, III and IV (Orsi et al. 2010). It has been observed that these lineages differ in their distribution and prevalence in the environment (Valderrama & Cutter 2013). Strains from lineage I are the most prevalent among human clinical isolates (Sauders & Wiedmann 2007), but are rare in food processing environments, where lineage II is more prevalent (Shank et al. 1996). Lineages III and IV are rare and more frequently associated with animals (Jeffers et al. 2001; Orsi et al. 2010).

Some researchers have tried to explain these associations based on the ability of the bacterium to form biofilms. Several studies suggest that lineage II is prevalent in food processing facilities because it has a higher ability to form biofilms. However, studies designed to address this hypothesis by ranking and comparing biofilm formation among *L. monocytogenes* strains under similar conditions have led to inconclusive and contradictory results. For instance, Borucki et al. (2003) and Lunden (2004) concluded that strains from lineage II are better able to form biofilms, compared with lineage I; the opposite has also been described elsewhere (Djordjevic et al. 2002; Takahashi et al. 2010), and others have claimed that lineages do not differ in their biofilm formation capacity (Milanov et al. 2009). These contradictions suggest that *L. monocytogenes* strains have lineage-associated environmental requirements for biofilm development. If this is true, comparison under the same set of conditions may favor some strains to the detriment of others and *vice versa*.

In the present study, the authors propose to reevaluate the paradigm of assessing the biofilm formation capacity of various lineages under similar conditions and apply a more comprehensive approach using multivariate analysis. This approach has been underexploited in the field of microbial ecology, despite its usefulness (Ramette 2007). The comparison of biofilm formation capacity among lineages under a wider array of conditions would help identify unique lineage-associated patterns. Adding this knowledge to epidemiological and environmental information would enhance the understanding of the ecology of *L. monocytogenes*.

The discussion of the distribution of *L. monocytogenes* from an ecological perspective requires the review of key concepts. An 'ecological niche' refers to a set of biotic (ie competitor species or predators) and abiotic environmental (ie temperature, availability of nutrients,

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and moisture) conditions in which a species is able to persist (Hutchinson 1957). In other words, abiotic conditions have the potential to determine the 'fundamental niche' and the biotic conditions determine the 'realized niche'. A hypothesis based on a microbial survey suggests that different phylogenetic lineages of *L. monocytogenes* have different but overlapping ecological niches, which affect the ability of the pathogen to enter the food chain (Sauders & Wiedmann 2007). However, experimental evidence of the existence of different fundamental or realized niches for *L. monocytogenes* lineages is still lacking.

Therefore, the present study aimed to demonstrate that environmental conditions affect the biofilm formation capacity of *L. monocytogenes* lineages (I, II and III) and to generate evidence that supports the hypothesis that these lineages have different but overlapping ecological niches.

Materials and methods

Bacterial strains

L. monocytogenes strains were obtained from the characterized collection of the PSU Food Microbiology Culture Collection, with the exception of some isolates donated by Dr Luke LaBorde, which were isolated from independent mushroom processing contamination events, and coded LLB-1 to LLB-6. One pair of each of four epidemic clones (EC) I to IV (each belonged to a different outbreak event) was included in the strain set (Table 1). The lineage of the mushroom-associated strains was confirmed previously by standard multiplex PCR procedures (Chen & Knabel 2007). Information from each strain (serotype, source, origin, and epidemiology) was gathered from the published literature in order to characterize the set of available strains for this study.

Culture preparation

Stock cultures of *L. monocytogenes* were stored at −80°C in tryptic soy broth (TSB; Difco, Detroit, MI, USA) with 10% glycerol (v/v). Working cultures were maintained on tryptic soy agar (TSA, Difco) following incubation at 37°C for 24 h. Plates were stored at 4°C for a maximum of 30 days. Prior to each experiment, a loopful of culture was transferred from the plates and grown in 9 ml of TSB supplemented with 6% yeast extract (TSBYE; Difco) and incubated at 35°C for 24 h. For this study, a total of 41 strains of *L. monocytogenes* were used.

Biofilm assessment

Biofilm formation was assessed by the microtiter plate method specifically developed for *L. monocytogenes* (Djordjevic et al. 2002) and evaluated in the laboratory. Among the advantages of the selected method are its capability to screen a large number of strains, reproducibility and a strong correlation with the microscopic evaluation of *L. monocytogenes* biofilms (Djordjevic et al. 2002; Borucki et al. 2003; Merritt et al. 2005). The selected set of abiotic conditions for this study were those likely to be found in environments of interest, such as food processing facilities, and included: nutrient availability, different pH values, water activity (a_w) and temperatures. For the purposes of this study, biofilm assessment was divided into two consecutive steps: biofilm development and biofilm quantification.

Biofilm development

In order to evaluate the effect of different, pre-established conditions on individual strains, distinctive culture media were developed. Each medium was made by aseptically combining TSBYE either with a brine solution (20% NaCl w/v), deionized water, or saline solution (0.8% NaCl w/v) to achieve the desired a_w measured at 25°C with an Aqua Lab 4TE water activity meter (Decagon Devices, Pullman, WA, USA), and adjusting the pH with either 5 N HCl or 5 N NaOH, as needed, using a SympHony SP-20 pH meter (VWR, West Chester, PA, USA). To confirm that a_w was responsible for the effect on biofilm capacity irrespective of the a_w modifier, a comparison was performed using KCl and glycerol (propan-1,2,3-triol) as alternatives to adjust the a_w of the growth media. The mean value of three different strains was evaluated independently in triplicate under four different water activity values (0.960, 0.970, 0.980, 0.997) and compared. The evaluation had the following fixed conditions: temperature at 30°C; TSBYE% at 30% v/v; and pH at 7.00 after 48 h incubation under static conditions. The obtained Pearson correlation coefficients between NaCl and KCl, NaCl and glycerol, and KCl and glycerol were r = 0.984, 0.908, and 0.935, respectively at $p \leq 0.05$. Since all the evaluated a_w modifiers had a similar effect, NaCl was chosen to adjust the a_w in the various media due to its low cost and availability.

Each well of a sterile, flat-bottomed, 96-well plate (Becton Dickinson Labware) was filled with 150 µl of TSBYE containing ~4 $\log_{10}$ CFU ml^{-1} of the pathogen. Uninoculated wells were used as negative controls and blanks. To minimize evaporative loss, plates were sealed with Parafilm™ (American National Can Company, Maryland, USA), and incubated for 48 h at respective temperatures under static conditions to allow biofilm development.

Biofilm and planktonic cell quantification

After incubation, media were removed and the microtiter plate wells were washed four times automatically with

200 µl of sterile, demineralized water, per rinse, using a Wellwash 4 MK2 (Thermo Fisher Scientific, Vantaa, Finland). To avoid undesired biofilm removal, washing nozzles were positioned 2.5 mm from the bottom of the plates. After rinsing, plates were air-dried in a biological safety hood and once dried, each well was stained with 100 µl of 1% crystal violet solution (Mallinckrodt Baker, Phillisburg, NJ, USA) for 45 min at room temperature. Excess crystal violet was removed by rinsing five times automatically with sterile, demineralized water following the same protocol described above. To quantify biofilm production, 150 µl of 95% ethanol were added to each well and left undisturbed for at least 10 min at room temperature. After destaining, 100 µl of the ethanol solution were transferred to a new microtiter plate and the optical density (OD) at 595 nm of the crystal violet present in each well was recorded using a Multiskan Spectrum Microtiter Plate Reader (Thermo Lab Systems, Virginia, USA).

The determination of planktonic cells was performed by transferring 100 µl of each well to a new plate for the measurement of the OD_{600} and subtracting the average OD_{600} from the negative control wells. Biofilm assessment was performed three times for all *L. monocytogenes* strains and the averages and standard deviations were calculated. The experiment was carried out following a blind design so that the identity and the coded information for each strain were uncovered for further data interpretation upon completion of the microtiter plate assays.

Experimental design and statistical analysis

The first phase of this study consisted of the construction of a data set based on biofilm formation by *L. monocytogenes* strains using the microtiter plate method in response to environmental conditions using a full factorial design. After determining the environmental conditions that affect biofilm formation, a cluster analysis of the mean values of the entire data set from each individual strain (n = 41) under each individual treatment (n = 81) was performed to determine whether a lineage-related response to the environmental factors could be detected. Finally, to identify which environmental factor or combinations affected biofilm formation, a construction of a linear regression equation for each individual strain, with a subsequent principal component analysis (PCA) of the regression coefficients, was devised. Only main effects and linear interactions were included in the regression model to facilitate interpretation.

Data set construction

The data set was built in two steps: first, the selection of the appropriate levels (3) for each environmental condition by means of the traditional one-factor-at-a-time (OFAT) approach; second, the construction of the database using a full factorial design (3^4) for each strain. Four experimental factors, temperature, nutrient availability (TSBYE%), a_w, and pH, were evaluated.

The authors are aware of the impact that other factors, such as surface material and characteristics, may have on biofilm formation by *L. monocytogenes*. This topic has been discussed extensively (Valderrama & Cutter 2013). However, in the present study, those factors were not included, due to the limitations of the microtiter plate assay.

Selection of appropriate levels for experimental factors

Seven randomly chosen strains were used (four lineage II, two lineage I, and one lineage III) for this experiment. Levels for each experimental factor were selected following the OFAT approach and based on the evaluated effect of an individual factor gradient after 48 h, while the other three factors remained fixed at optimum values as follows: a_w = 0.994, TSBYE% = 30% v/v, temperature = 30°C, and pH = 7. Factor levels were tested using a range of incubation temperatures (15, 20, 25, 30, 35, 40°C), TSBYE% (0, 5, 10, 20, 30, 60, 80, 100 v/v), a_w (0.938, 0.955, 0.968, 0.978, 0.983, 0.988, 0.993, 0.998), and pH (4, 5, 6, 7, 8, 9) levels. Each condition, with the exception of temperature, was achieved as described previously.

Full factorial

A 3^4 full factorial design was carried out to determine the individual effects of temperature, TSBYE %, a_w and pH and their interactions on biofilm formation. Minitab version 16 (State College, PA, USA) was used for regression and graphic analyses of the data. Three replicates were performed for the 81 runs, for a total of 243 data points per each strain (n = 41).

For this experiment, bacterial cultures individually grown in full strength TSBYE for 24 h at 35°C were serially diluted in saline solution to achieve ~10^7 CFU ml^{-1}, and 10 µl transferred to a 96 deep-well plate (VWR, Radnor, PA, USA) containing 1990 µl of medium under appropriate conditions. The 96 deep-well plate served as a template to make identical replicates stored at the selected three different temperatures (20, 25, 30°C). From the 96 deep-well plate, 150 µl were transferred to each well of a flat-bottomed, polystyrene 96 well plate (Becton Dickinson Labware; Franklin Lakes, NJ, USA) containing 150 µl of the corresponding treatment. The initial inoculum in each well was ~10^4 CFU ml^{-1}.

Cluster analysis

The cluster analysis was carried out using Minitab (version 16) software, with Euclidean distances and the

Table 1. General information and characteristics of the 41 representative strains used in this study.

Strain name	Lineage	Serotype	Ribotype	Source	Origin	Epidemic clone, outbreak	Epidemiology	Reference
FSL J1–126	I	4b	DUP-1038B	Human	CH	EC I, cheese, 1983–1987	Epidemic	(Food microbe Tracker 2003–2013)
FSL J1–003	I	4b	DUP-1038	Human	CA	EC I, coleslaw, 1981	Epidemic	(Chen et al. 2007)
H3396	I	4b	DUP-1044	NA	USA	EC II, hot dog, 1998	Epidemic	(Chen et al. 2007)
J2685	I	4b	DUP-1044A	NA	USA	EC II, deli. meat, 2002	Epidemic	(Chen et al. 2007)
FSL J1–129	I	4b	DUP-1042B	Human	UK	EC IV, pate, 1989	Epidemic	(Chen et al. 2007)
FSL J1–220	I	4b	DUP-1042	Human	USA	EC IV, vegetable, 1979	Epidemic	(Chen et al. 2007)
ATCC 19115	I	4b	NA	Human	USA		NA	(Chen et al. 2007)
FSL R2–502	I	1/2b	DUP-1051B	Food	USA		Epidemic	(Chen et al. 2007)
FSL F2–239	I	1/2b	DUP1042C	Food	USA		Sporadic	(Chen et al. 2007)
FSL J1–225	I	4b	DUP-1042B	Human	USA		Epidemic	(Chen et al. 2007)
FSL R2–501	I	4b	DUP-1042B	Human	USA		Epidemic	(Chen et al. 2007)
FSL C1–122	I	4b	DUP-1038B	Human	USA		Sporadic	(Chen et al. 2007)
FSL J2–044	I	4b	DUP-1042	Primate	USA		Sporadic	(Chen et al. 2007)
FSL N3–010	I	4b	NA	Food	UK		Sporadic	(Chen et al. 2007)
FSL F2–293	I	1/2b	DUP1031A	Food	USA		Sporadic	(Food microbe Tracker 2003–2013)
FSL J1–049	I	3C	DUP1042C	Human			Sporadic	(Food microbe Tracker 2003–2013)
FSL J1–169	I	3b	DUP-1052A	Human	USA		Sporadic	(Food microbe Tracker 2003–2013)
FSL J1–177	I	1/2b	DUP-1051D	Human	USA		Sporadic	(Food microbe Tracker 2003–2013)
FSL J1–012	I	4b	DUP-1038B	Human	USA		Epidemic	(Food microbe Tracker 2003–2013)
FSL N1–011A	I	1/2b	NA	Food environment			Epidemic	(Chen et al. 2007)
FSL F2–525	I	4b	DUP-1061A	Human			Sporadic	(Chen et al. 2007)
FSL S4–436	I	1/2b	DUP-1025A	Non-food environment			Sporadic	(Chen et al. 2007)
FSL R2–603	II	1/2a	DUP-1053A		USA	EC III, deli. meat 2000	Epidemic	(Chen et al. 2007)
FSL N3–031	II	1/2a	DUP-1053A	Food	USA	EC III, hot dog 1989	Sporadic	(Chen et al. 2007)
FSL J1–094	II	1/2c	NA	Human	UK		Sporadic	(Chen et al. 2007)
FSL C1–115	II	3a	NA	Human	USA		Sporadic	(Chen et al. 2007)
FSL F2–373	II	1/2a	DUP-1039C	Food			Sporadic	(Chen et al. 2007)
FSL N1–014	II	1/2a	NA	Food			Sporadic	(Chen et al. 2007)
LLB-1	II	1/2a	NA	Food	USA		Sporadic	*
LLB-2	II	1/2a	NA	Food	USA		Sporadic	*
LLB-3	II	1/2a	NA	Food	USA		Sporadic	*
LLB-4	II	1/2a	NA	Food	USA		Sporadic	*
LLB-5	II	1/2a	NA	Food	USA		Sporadic	*
LLB-6	II	1/2a	NA	Food	USA		Sporadic	*
FSL J1–105	II	1/2a	DUP-1030	Human			Epidemic	(Chen et al. 2007)
FSL N4–588	II	NA	DUP-1045B	Non-food environment	USA		Sporadic	(Chen et al. 2007)
FSL L3–151	II	1/2a	DUP-1039A	Food environment			Sporadic	(Chen et al. 2007)
FSL R2–499	II	1/2a	DUP-1053A	Human			Epidemic	(Chen et al. 2007)

(*Continued*)

Table 1. (*Continued*).

Strain name	Lineage	Serotype	Ribotype	Source	Origin	Epidemic clone, outbreak	Epidemiology	Reference
FSL J1–101	II	1/2a	DUP-1053A	Human			Epidemic	(Chen et al. 2007)
FSL J1–031	III	4a	DUP-1059A	Human			Sporadic	(Food microbe Tracker 2003–2013)
FSL J1–168	III	4a	116–110-S-2	Human			Sporadic	(Food microbe Tracker 2003–2013)
FSL F2–695	III	4a	DUP-1061A	Human			Sporadic	(Food microbe Tracker 2003–2013)
FSL F2–655	III	NA	116–110-S-2	Human			Sporadic	(Food microbe Tracker 2003–2013)

Note: NA: not available.
*strains found in mushrooms and obtained from Dr .Luke LaBorde
Ribotyping pattern designations followed by a capital letter indicate subribotype (eg DUP-1044A is a subribotype of ribogroup DUP-1044)

Ward hierarchical method. The single, complete, average, or median linkage methods displayed similar results.

Multiple linear regression and principal component analysis

A multiple linear regression was performed for each strain in order to reduce the number of explanatory variables. Equation 1 describes the linear regression model as a response function of the environmental factors:

$$\begin{aligned} Y_{595} = {} & \propto_0 + \propto_1 X_1 + \propto_2 X_2 + \propto_3 X_3 + \propto_4 X_4 \\ & + \propto_5 X_1X_2 + \propto_6 X_1X_3 + \propto_7 X_1X_4 + \propto_8 X_2X_3 \\ & + \propto_9 X_2X_4 + \propto_{10} X_3X_4 + \propto_1 X_1X_2X_3X_4 \\ & + \propto_{12} X_1X_2X_4 + \propto_{13} X_1X_3X_4 + \propto_{14} X_2X_3X_4 \\ & + \propto_{15} X_1X_2X_3X_4 \end{aligned} \quad (1)$$

where Y_{595} (OD_{595}) is the measured response associated with each factor combination; α_0 to α_{15} are the regression coefficients; and X1, X2, X3, and X4 are the factors. The principal component analysis (PCA) of these regression coefficients from the linear regression of each strain was carried out using the Minitab statistical package version 16 by means of the correlation matrix approach.

Results and discussion

Strain collection

The set of strains obtained for this study is described in Table 1. The profile of the strain collection was as follows: 21 belong to lineage I; 15 to lineage II; and four to lineage III. Lineage I is predominant in culture collections due to its high association with human illnesses and thus, higher numbers of this lineage strain were obtained for this study. The reduced number of strains belonging to lineage III was expected and is in agreement with the scarcity of this lineage in microbial collections.

Regarding sample size, a few studies comparing biofilm formation between lineages have used a larger number of strains (>100) (Norwood & Gilmour 1999; Borucki et al. 2003; Harvey et al. 2007) while others had used smaller (<40 strains) sample sizes (Lunden et al. 2000; Kalmokoff et al. 2001; Djordjevic et al. 2002; Folsom et al. 2006; Milanov et al. 2009). Thus, the strain set of 41 used in the present study was considered adequate and representative of the diversity among *L. monocytogenes* strains.

Environmental conditions influencing biofilm formation

Figure 1 demonstrates biofilm formation and growth of planktonic cells under different conditions of temperature, TSBYE%, a_w and pH. Identification of favorable conditions for biofilm development has received little attention since it has been assumed that the same optimum growth conditions for planktonic cells enhance biofilms. The results of the OFAT approach demonstrate that temperature, TSBYE%, a_w and pH have an independent effect on biofilm and planktonic cells (Figure 1). Maximum absorbance levels were different for biofilm and planktonic cells. Ranges of temperature of 30–35°C, TSBYE% of 10–20% v/v, a_w of 0.978–0.988, and pH values of 7–8 yielded maximum absorbance for biofilms. For all evaluated strains, biofilms exhibited a sigmoid curve as a function of TSBYE%, a_w, and pH. When the effect of temperature was evaluated individually, lineages I and II demonstrated a distinct behavior. All strains from lineage I showed maximum biofilm development at 30°C, while lineage II exhibited maximum biofilm production at 35°C (Figure 1, left). These results support the hypothesis that *L. monocytogenes* lineages (I *vs* II) respond to environmental conditions differently when forming biofilms. Significant differences ($p < 0.05$) in biofilm formation were observed at 25°C and 30°C between lineages I and II, while lineage III was similar

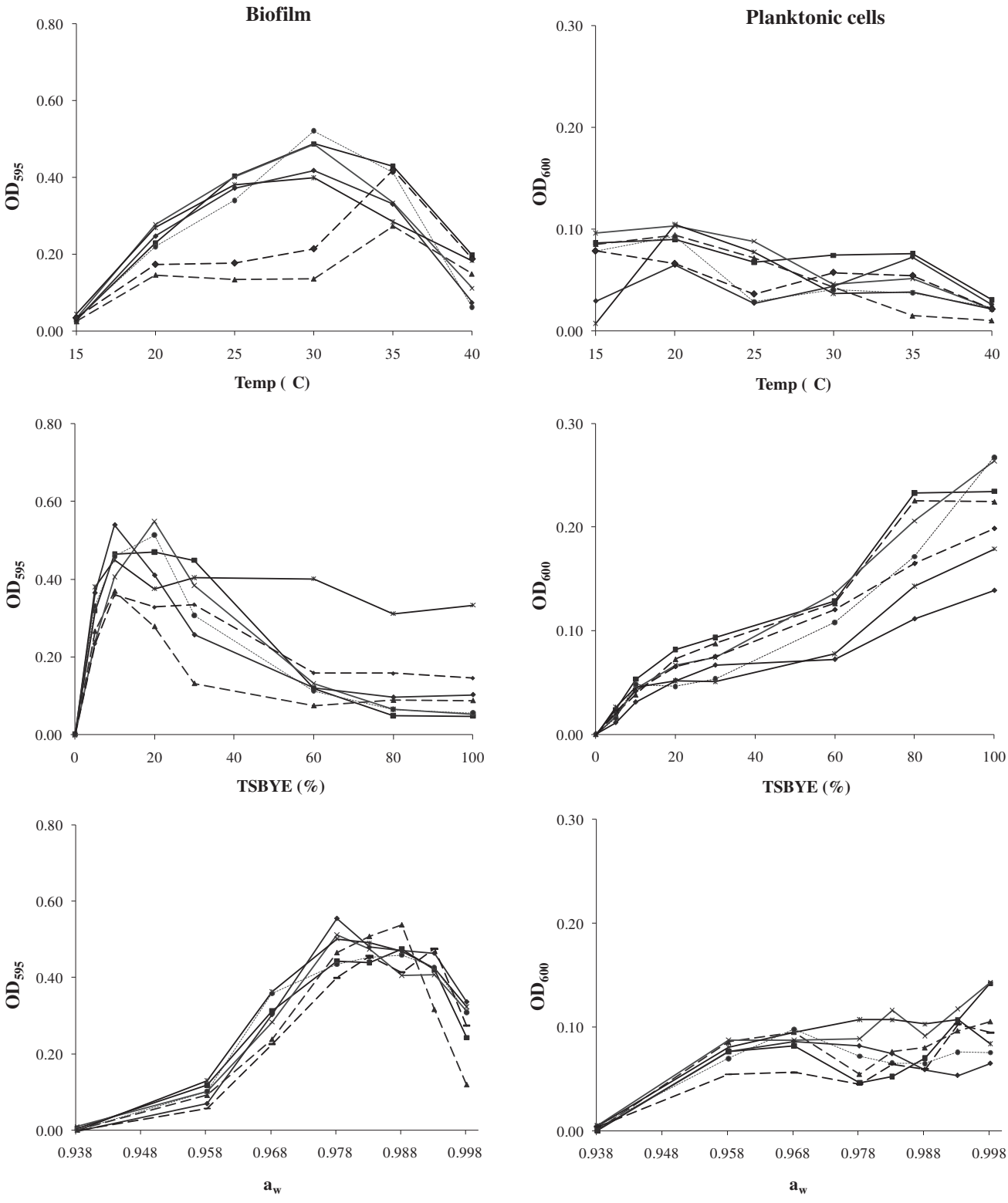

Figure 1. Effect of environmental factors on biofilm formation (right) and planktonic cells (left) expressed as the mean OD of seven strains of *L. monocytogenes* belonging to lineages I (—), II (–) and III (…) under fixed conditions: temperature 30°C; 30% v/v TSBYE%; 0.99 aw and pH 7.00 after incubation for 48 h.

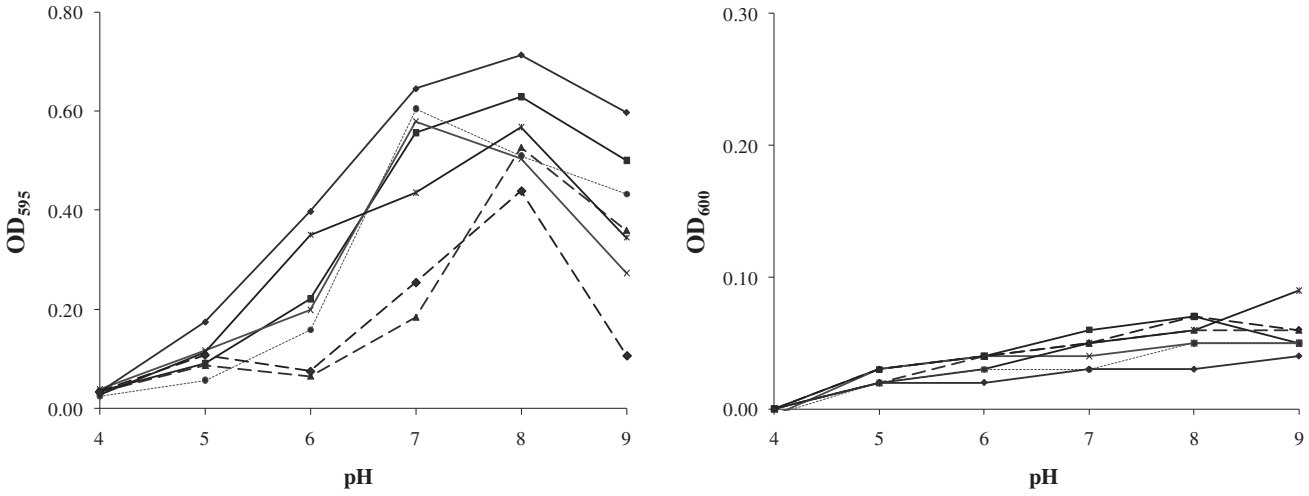

Figure 1. (*Continued*).

to lineage I under similar conditions. Comparable results were observed when pH was evaluated; OD values exhibited a high variability at pH 7, demonstrating that strains from lineages I and II have different biofilm development patterns. Finally, when the effect of nutrient availability was evaluated, TSBYE% < 30% (ie limited nutrient availability) resulted in a peak of biofilm formation for all strains and agreed with other studies (Hood & Zottola 1995, 1997).

Growth patterns of planktonic cells as a function of the evaluated conditions are observed on the right of Figure 1. For all strains, a temperature of 20°C favored maximum biofilm formation. When TSBYE% was modified, a positive relationship was observed for all strains so that an increase of nutrient availability boosted the number of planktonic cells. As expected, cell growth occurred only at pH values > 4, and was enhanced at a_w > 0.96.

A comparison between the conditions that favored biofilm development and those that enhanced planktonic growth reveals that the effect of environmental factors is completely different and even opposite, especially for TSBYE% and temperature (see Figure 1). This result is particularly relevant for understanding the ability of *L. monocytogenes* to grow and survive under certain conditions. The traditional approach has been to assess free-living bacteria (planktonic), although it is widely recognized that most bacteria are found as biofilms (Davey & O'Toole 2000). The present study demonstrates that the conditions that enhance growth of planktonic cells differ from those that favor biofilm formation and supports the approach of assessing biofilm formation in order to understand the pathways of colonization of specific environments by *L. monocytogenes*.

The OFAT analysis also allowed for the identification of environmental conditions that reveal differences between lineages. These levels are summarized in Table 2 and were used to perform the full factorial, cluster and PCA analysis.

Relationship between lineages and biofilm patterns

The results from the cluster analysis are summarized in Figure 2. The mean OD_{595} values obtained from the biofilm assessment of the 41 strains, in response to 81 different conditions, were used to obtain the observed clusters. The dendrogram revealed different biofilm development patterns grouped according to their similarity level. At 80% similarity, five clusters, based on pathogen lineage, are revealed (Table 3). The other, so called 'label information' of the strains (serotype, ribotype, and origin), was unable to provide identifiable clusters at any level of similarity (data not shown).

Considering the definition of fundamental niche as the set of abiotic or environmental requirements for a given organism, 'fitness' can be defined as the favorable or unfavorable response of that organism to a specific set of conditions. Theoretically, the amount of biofilm produced by two or more bacteria under a specific set of environmental conditions can provide an estimate of the degree of fitness (Valderrama & Cutter 2013). Thus, the knowledge of an organism's niche is essential for

Table 2. Levels of factors chosen for the experimental design.

Factors	Variable	Levels		
Temp (°C)	X_1	20	25	30
TSBYE% v/v	X_2	20	40	60
a_w	X_3	0.96	0.97	0.98
pH	X_4	6	7	8

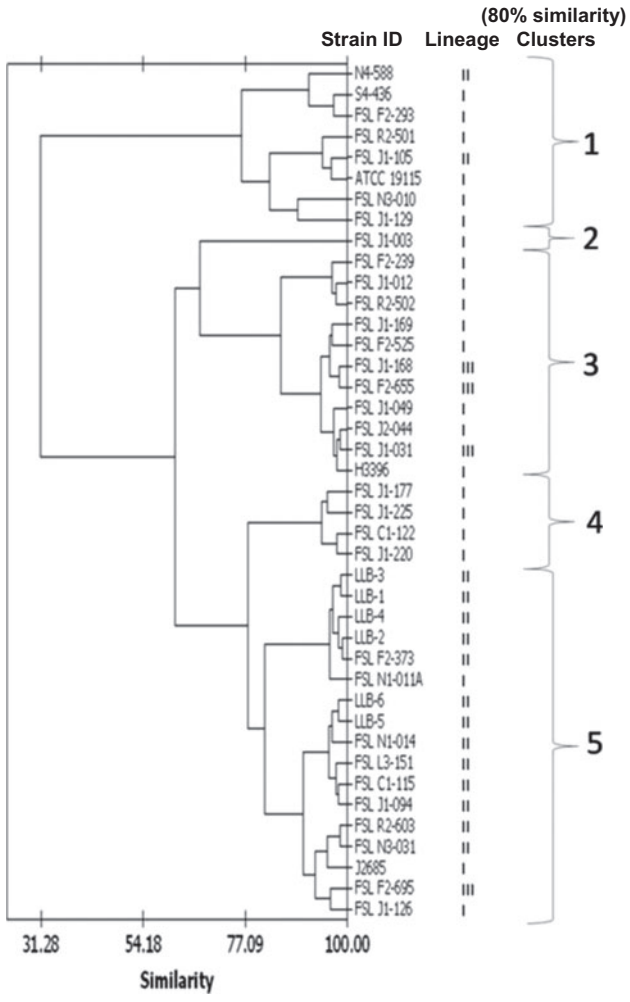

Figure 2. Dendrogram of the 41 *L. monocytogenes* strains based on biofilm formation in response to 81 different conditions using Ward's hierarchical lineage method.

Table 3. Distribution of lineages of *L. monocytogenes* and total strains comprising each cluster obtained by performing Ward's hierarchical clustering of the absorbance values (OD_{595}) at 80% similarity level.

	Lineage			
Cluster	**I**	**II**	**III**	**Total strains**
1	6	2	0	8
2	1	0	0	1
3	8	0	3	11
4	4	0	0	4
5	3	13	1	17
Total strains	22	15	4	41

understanding and even predicting its geographic distribution (Polechová & Storch 2008). The evaluation of biofilm formation under a wide variety of environmental conditions allows the comparison of patterns between two or more species. If the microorganisms have similar biofilm patterns, they may share the same fundamental niche, and potentially, a similar ecological niche.

The distribution of *L. monocytogenes* strains among the clusters (see Figure 2) demonstrate that, irrespective of the variability and high degree of similarity (80%), common biofilm patterns and lineage-related clusters can be distinguished. For instance, strains belonging to lineage II predominate in cluster 5 (see Table 3), strains from lineage I were present in every cluster, and strains from lineage III were grouped in only two clusters (1 and 5).

These results demonstrate that lineage II is less variable in its ability to form biofilm, as compared with lineage I. This finding supports the hypothesis that lineage II may have adapted to a specific environment, such as food processing facilities (Sauders & Wiedmann 2007), where abiotic conditions (ie temperature, pH) are closely monitored, and therefore more stable, as compared with natural environments.

The cluster analysis also revealed that lineages I and III have indistinguishable biofilm formation patterns, probably because these lineages are genetically related (Nightingale et al. 2005). The results also may support the hypothesis that *L. monocytogenes* lineages I and II have different but overlapping ecological niches. Lineage I has been described as 'highly clonal', and lineage II as having greater genetic diversity (Nightingale et al. 2005). This diversity may explain why this lineage would exhibit greater biofilm formation versatility in response to environmental factors. However, the cluster analysis reveals the opposite.

Finally, the cluster analysis in terms of epidemic clones demonstrate that strains belonging to EC III have a fundamental niche different from EC I, II, and IV, which were grouped in different clusters (Table 4). These results suggest that, despite their common ancestor, these EC do not share biofilm patterns, have different fundamental niches, and thus may differ in their contamination pathways.

Table 4. Distribution of epidemic clones of *L. monocytogenes* contained in each cluster obtained by performing Ward's hierarchical clustering of the absorbance values (OD_{595}) at 80% similarity level.

	Epidemic clones				
Cluster	**EC-I**	**EC-II**	**EC-III**	**EC-IV**	**Total EC**
1				1	1
2	1				1
3		1			1
4				1	1
5	1	1	2		4
Total EC	2	2	2	2	8

Multiple linear regression and principal component analysis

To further identify environmental factors responsible for biofilm formation, a PCA was performed using the coefficients obtained from the multiple linear regression analysis (data not shown) obtained for each isolate. The first two PC with eigenvalues >1 explained the 88% of the total variance in biofilm formation and are plotted in Figure 3. In order to interpret this figure the highest positive and negative loading factors (Table 5) for each PC_x were selected to plot the 41 evaluated strains.

The highest positive and negative loading factors for PC_1 (0.30, −0.30) were associated with temperature and the interaction TSBYE%*pH, respectively. This observation suggests the presence of two sub-groups, across all the evaluated lineages with an unknown common data label. PC_2 exhibited a high positive loading factor (0.40) associated with the interaction temperature*a_w*pH, and a high negative loading factor (−0.38) associated with the interaction of a_w*pH. Only PC_2 was effective in distinguishing phylogenetic lineages (see Figure 3), such that the upper section of the plot was dominated by strains belonging to lineage II, with some overlapping strains of lineages I and III. High temperature, a_w and pH values favor lineage II biofilm formation, while lineages I and III have lower biofilm capacity at high a_w and pH values. In general, high nutrient availability negatively affects biofilm formation, but this is particularly true for lineages I and III.

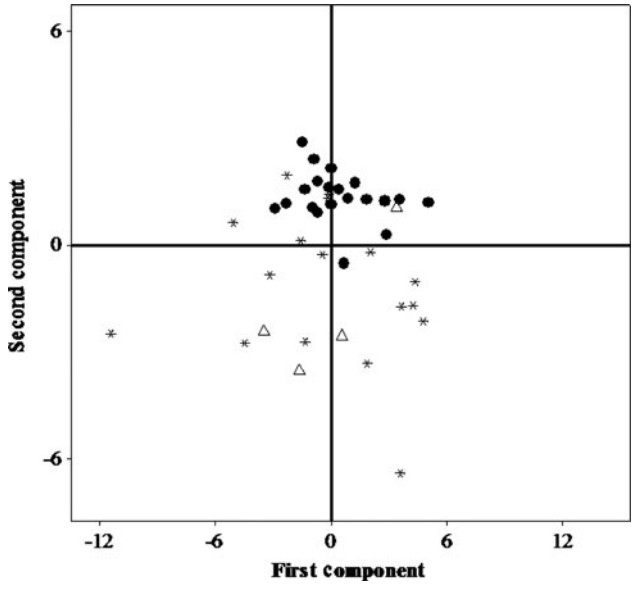

Figure 3. Score plot for PCA of biofilm formation data from 41 *L. monocytogenes* strains. * = lineage I; ● = lineage II; △ = lineage III.

Table 5. Loading factors obtained from the PCA analysis of the coefficients obtained from the multiple linear regression of each strain.

	Principal components	
Factors	**PC1**	**PC2**
Constant	−0.27	0.19
Temperature (Temp)	0.30	−0.09
TSBYE%	0.23	−0.32
a_w	0.28	−0.13
pH	0.23	0.23
Temp*TSBYE%	−0.26	0.25
Temp*a_w	−0.27	0.11
Temp*pH	−0.21	−0.33
TSBYE*a_w	−0.25	0.26
TSBYE% *pH	−0.30	0.01
a_w *pH	−0.18	−0.38
Temp*TSBYE% *a_w	0.26	−0.24
Temp*TSBYE% *pH	0.28	0.05
Temp*a_w *pH	0.17	0.40
TSBYE%*a_w *pH	0.25	0.27
Temp*TSBYE%*a_w *pH	−0.23	−0.32

These results suggest that temperature might be the key factor responsible for differential fitness between *L. monocytogenes* lineages. For instance, in food processing facilities where abiotic conditions, such as temperature, are controlled and predictable, lineages positively affected by this factor may have an advantage in forming biofilms in comparison with lineages with lower ability to use temperature as an asset to colonize the same environment. Although these results provide some insights, future research in the field is needed to confirm this statement through the collection of environmental information associated with positive *L. monocytogenes* samples collected at food processing facilities.

The present study demonstrates that multivariate analysis is more powerful than a side-by-side comparison of individual conditions to evaluate biofilm formation capacity among strains and to provide sufficient evidence to differentiate lineages. The cluster and PCA analysis demonstrate that *L. monocytogenes* strains have individual environmental requirements related to their lineage and, thus, different fundamental ecological niches. The data also support the hypothesis that the prevalence of the pathogen in a determined niche (eg food processing facilities) may be influenced by the existing environmental factors. While further research is needed to confirm this statement, the results demonstrate the importance of understanding the influence of environmental factors on biofilm formation, transmission pathways and true reservoirs of *L. monocytogenes* (Valderrama & Cutter 2013). Nevertheless, this study provides a framework for future studies on the environmental specificity and transmission characteristics of

different *L. monocytogenes* strains and lineages. The amount of biofilm formation has the potential to be used as a fitness indicator (Valderrama & Cutter 2013) and provide some rationale for the different prevalence of certain microorganisms in specific habitats.

Acknowledgements

This research was funded by a USDA-Milk Safety Grant and through the Department of Food Science, College of Agricultural Sciences at the Pennsylvania State University. The authors also thank Drs Robert Roberts and Gregory Ziegler for their guidance and support of the project.

References

Barbalho T, Almeida PF, Almeida RCC, Hofer E. 2005. Prevalence of *Listeria* spp. at a poultry processing plant in Brazil and a phage test for rapid confirmation of suspect colonies. Food Control. 16:211–216.

Beumer RR, Giffel MCT, Spoorenberg E, Rombouts FM. 1996. *Listeria* species in domestic environments. Epidemiol Infect. 117:437–442.

Borucki MK, Peppin JD, White D, Loge F, Call DR. 2003. Variation in biofilm formation among strains of *Listeria monocytogenes*. Appl Environ Microbiol. 69:7336–7342.

Chao G, Deng Y, Zhou X, Xu Q, Qian X, Zhou L, Zhu B. 2006. Prevalence of *Listeria monocytogenes* in delicatessen food products in China. Food Control. 17:971–974.

Chen Y, Knabel SJ. 2007. Multiplex PCR for simultaneous detection of bacteria of the genus *Listeria*, *Listeria monocytogenes*, and major serotypes and epidemic clones of *L. monocytogenes*. Appl Environ Microbiol. 73: 6299–6304.

Chen Y, Zhang W, Knabel SJ. 2007. Multi-virulence-locus sequence typing identifies single nucleotide polymorphisms which differentiate epidemic clones and outbreak strains of *Listeria monocytogenes*. J Clin Microbiol. 45:835–846.

Davey ME, O'Toole GA. 2000. Microbial biofilms: from ecology to molecular genetics. Microbiol Mol Biol Rev. 64:847–867.

Djordjevic D, Wiedmann M, McLandsborough LA. 2002. Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. Appl Environ Microbiol. 68:2950–2958.

Doorn J, Medeiros L, Lejeune J, Kersting A, Kendall P, Schroeder M, Sofos J, Rodriguez-Marval M. 2007. Household member behaviors and prevalence of *Listeria monocytogenes* in the home environment. J Nutr Educ Behav. 39:S115–S115.

Fenlon DR. 1986. Growth of naturally occurring *Listeria* spp. in silage: a comparative study of laboratory and farm ensiled grass. Grass Forage Sci. 41:375–378.

Folsom J, Siragusa G, Frank J. 2006. Formation of biofilm at different nutrient levels by various genotypes of *Listeria monocytogenes*. J Food Prot. 69:826–834.

Food Microbe Tracker (previously Pathogen Tracker 2.0). The Cornell Food Safety Laboratory Bacterial Strains WWW Database Project. 2003–2013. New York: Cornell University. Available from: http://www.foodmicrobetracker.com/.

Guerini MN, Brichta-Harhay DM, Shackelford SD, Arthur TM, Bosilevac JM, Kalchayanand N, Wheeler TL, Koohmaraie M. 2007. *Listeria* prevalence and *Listeria monocytogenes* serovar diversity at cull cow and bull processing plants in the United States. J Food Prot. 70:2578–2582.

Harvey J, Keenan KP, Gilmour A. 2007. Assessing biofilm formation by *Listeria monocytogenes* strains. Food Microbiol. 24:380–392.

Hood SK, Zottola EA. 1995. Biofilms in food processing. Food Control. 6:9–18.

Hood SK, Zottola EA. 1997. Adherence to stainless steel by foodborne microorganisms during growth in model food systems. Int J Food Microbiol. 37:145–153.

Hutchinson GE. 1957. A treatise on limnology. New York: Wiley.

Jeffers GT, Bruce JL, McDonough PL, Scarlett J, Boor KJ, Wiedmann M. 2001. Comparative genetic characterization of *Listeria monocytogenes* isolates from human and animal listeriosis cases. Microbiology. 147:1095–1104.

Kalmokoff ML, Austin JW, Wan XD, Sanders G, Banerjee S, Farber JM. 2001. Adsorption, attachment and biofilm formation among isolates of *Listeria monocytogenes* using model conditions. J Appl Microbiol. 91:725–734.

Lunden J. 2004. Persistent *Listeria monocytogenes* contamination in food processing plants [dissertation]. Finland: University of Helsinki.

Lunden J, Miettinen M, Autio T, Korkeala H. 2000. Persistent *Listeria monocytogenes* strains show enhanced adherence to food contact surface after short contact times. J Food Prot. 63:1204–1207.

Lyautey E, Lapen DR, Wilkes G, McCleary K, Pagotto F, Tyler K, Hartmann A, Piveteau P, Rieu A, Robertson WJ, et al. 2007. Distribution and characteristics of *Listeria monocytogenes* isolates from surface waters of the South Nation River watershed, Ontario, Canada. Appl Environ Microbiol. 73:5401–5410.

Merritt JH, Kadouri DE, O'Toole GA. 2005. Growing and analyzing static biofilms. Curr Protoc Microbiol. 1B.1:1B.1.1–1B.1.17.

Milanov D, Ašanin R, Vidić B, Katić V, Plavša N. 2009. Examination of the capabilities of attachment and biofilm formation of different *Listeria monocytogenes* strains. Biotech Anim Husbandry. 25:1255–1265.

Nightingale KK, Windham K, Wiedmann M. 2005. Evolution and molecular phylogeny of *Listeria monocytogenes* isolated from human and animal listeriosis cases and foods. J Bacteriol. 187:5537–5551.

Norwood DE, Gilmour A. 1999. Adherence of *Listeria monocytogenes* strains to stainless steel coupons. J Appl Microbiol. 86:576–582.

Oliver HF, Wiedmann M, Boor KJ. 2007. Environmental reservoir and transmission into the mammalian host. In: Goldfine H, Hao Shen H., editors. *Listeria monocytogenes*: pathogenesis and host response. New York: Springer; p. 111–137.

Orsi RH, Bakker HCd, Wiedmann M. 2010. *Listeria monocytogenes* lineages: genomics, evolution, ecology, and phenotypic characteristics. Int J Med Microbiol. 301:79–96.

Polechová J, Storch D. 2008. Ecological niche. In: Jorgensen SE, Fath B, editors. Encyclopedia of ecology. Oxford: Elsevier; p. 1088–1097.

Ramette A. 2007. Multivariate analyses in microbial ecology. FEMS Microbiol Ecol. 62:142–160.

Sauders BD, Wiedmann M. 2007. Ecology of *Listeria monocytogenes* and *Listeria* species in the natural environment. In: Ryser ET, Marth EH, editors. *Listeria*, listeriosis, and food safety. 3rd ed. Boca Raton (FL): CRC Press; p. 21–44.

Sergelidis D, Abrahim A, Sarimvei A, Panoulis C, Karaioannoglou P, Genigeorgis C. 1997. Temperature distribution and prevalence of *Listeria* spp. in domestic, retail and industrial refrigerators in Greece. Int J Food Microbiol. 34:171–177.

Shank FR, Elliot EL, Wachsmuth IK, Losikoff ME. 1996. US position on *Listeria monocytogenes* in foods. Food Control. 7:229–234.

Takahashi H, Suda T, Tanaka Y, Kimura B. 2010. Cellular hydrophobicity of *Listeria monocytogenes* involves initial attachment and biofilm formation on the surface of polyvinyl chloride. Lett Appl Microbiol. 50:618–625.

Valderrama WB, Cutter CN. 2013. An ecological perspective of *Listeria monocytogenes* biofilms in food processing facilities. Crit Rev Food Sci Nutr. 53:801–817.