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Variations in Biofilm Formation, Desiccation Resistance and Benzalkonium Chloride Susceptibility among *Listeria monocytogenes* Strains Isolated in Canada

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Abstract

Listeria monocytogenes is a pathogenic foodborne microorganism noted for its ability to survive in the environment and food processing facilities. Survival may be related to the phenotype of individual strains including the ability to form biofilms and resist desiccation and/or sanitizer exposure. The objectives of this research were to compare 14 *L. monocytogenes* strains isolated from blood (3), food (6) and water (5) with respect to their benzalkonium chloride (BAC) sensitivity, desiccation resistance, and ability to form biofilm. Correlations were tested between those responses, and the presence of the Stress Survival Islet (SSI-1) and Listeria Genomic Island 1 in a clonal complex 8 background (LGI1/CC8) genetic markers. Genetic sequences from four strains representing different phenotypes were also probed for predicted amino acid differences in biofilm, desiccation, and membrane related genes. The water isolates were among the most desiccation susceptible strains, while strains exhibiting desiccation resistance harboured SSI-1 or both the SSI-1 and LGI1/CC8 markers. BAC resistance was greatest in planktonic LGI1/CC8 cells (relative to non-LGI1/CC8 cells), and higher BAC concentrations were also needed to inhibit the formation of biofilm by LGI1/CC8 strains during incubation for 48 h and 6 days compared to other strains. Formation of biofilm on stainless steel was not significantly (p>0.05) different among the strains. Analysis of genetic sequence data from desiccation and BAC sensitive (CP4 5-1, CP5 2-3, both from water), intermediate (Lm568, food) and desiccation and BAC resistant (08 5578, blood, human outbreak) strains led to the finding of amino acid differences in predicted functional protein domains in several biofilm, desiccation and peptidoglycan related genes (e.g., *lmo0263*, *lmo0433*, *lmo0434*, *lmo0771*, *lmo0973*, *lmo1080*, *lmo1224, lmo1370*, *lmo1744,* and *lmo2558*). Notably, the LGI1/CC8 strain 08-5578 had a frameshift mutation in *lmo1370*, a gene previously associated with desiccation

resistance. In conclusion, the more desiccation and BAC resistant LGI1/CC8 isolates may pose a

challenge for sanitation efforts.

1.0 Introduction

Listeria monocytogenes is a pathogenic bacterium that causes the foodborne illness listeriosis (Donnelly, 2001). The pathogen is capable of adhering to and subsequently forming biofilms, which is known to enhance the resistance of cells to sanitizers, or hinder physical removal (Carpentier and Cerf, 2011; Lourenço et al., 2011). *L. monocytogenes* may enter food processing facilities via agricultural commodities (Todd and Notermans, 2011), or water (Weller et al., 2015), however, contamination of foods during processing is thought to lead to the majority of *L. monocytogenes* outbreaks (Oliver et al., 2007).

An individual strain of *L. monocytogenes* can be considered persistent if it is isolated from the same food processing facility over a period of several months or years (Ferreira et al., 2014; Magalhães et al., 2016). It may be hypothesized that persistence is related to the strain's ability to survive and reproduce under various environmental conditions. Among characteristics thought to be essential for survival in the processing environment is biofilm formation, where some studies report that persistent strains have better initial attachment to surfaces (cells/cm^2) and early biofilm formation, but do not to exhibit significant differences in biofilm formation during longer incubation periods as compared to transient or non-persistent strains (Lundén et al., 2000; Wang et al., 2015). In contrast, Ochiai et al. (2014) demonstrated persistent *L. monocytogenes* strains formed more biofilm than non-persistent strains, but this was temperature dependent. Meanwhile, Koreňová et al. (2016) and Costa et al. (2016) found no differences in biofilm formation between persistent and non-persistent strains, while Nilsson et al. (2011) showed many persistent strains formed less biofilm than non-persistent *L. moncytogenes* strains. Taken together it appears that biofilm formation varies widely among strains.

Sanitizer susceptibility is another important characteristic where differences have been reported among planktonic *L. monocytogenes* cells from persistent and non-persistent strains (Aase et al., 2000). In contrast, other researchers found no differences in sanitizer susceptibility between persistent and non-persistent planktonic cells (Harvey et al., 2007; Holah et al., 2002). The literature similarly contains opposing observations when it comes to sanitizer sensitivity of biofilms, as many workers (Costa et al., 2016; Kastbjerg and Gram, 2009; Wang et al., 2015) found no differences in sanitizer susceptibility of biofilms made by persistent and non-persistent *L. monocytogenes* strains, while Nakamura et al. (2013) reported finding significant differences.

Other mechanisms such as desiccation survival should be considered when evaluating the survival potential of *L. monocytogenes* in food plants (Burgess et al., 2016; Carpentier and Cerf, 2011). *L. monocytogenes* can survive desiccation for weeks or months on stainless steel surfaces (Takahashi et al., 2011; Vogel et al., 2010) and its desiccation survival is improved by biofilm formation (Hingston et al., 2013).

In search of genetic markers that predict the hardiness of *L. monocytogenes,* Ryan et al. (2010) discovered the stress survival islet (SSI-1), which contains a cluster of genes conferring bile tolerance and acid resistance leading to improved cold growth and survival during exposure to acid and osmotic stress. Later studies could, however, neither link SSI-1 to persistence as it was found to be both present and absent in persistent strains (Holch et al., 2013), nor to increased biofilm formation or benzalkonium chloride (BAC) resistance (Ebner et al., 2015). The *Listeria* Genomic Island 1 (LGI1) is a 50 kbp horizontally acquired genetic feature that has been detected in clinical and food processing *L. monocytogenes* strains belonging to the Clonal Complex 8 (CC8) (Gilmour et al., 2010). It encodes a putative peptidoglycan hydrolase, an adhesin, which

may influence biofilm formation, as well as type IV secretion system genes, and a multidrug efflux pump (Gilmour et al., 2010).

One study has implicated the involvement of CC8 strains in many listeriosis outbreaks in Canada during the last 30 years, and it was suggested that strains belonging to this complex might be particularly successful both as a human pathogen and in other environmental niches (Knabel et al., 2012). Interestingly, Verghese et al. (2011) reported that a CC8 strain formed more biofilm than other strains also isolated from meat and poultry processing facilities in Canada.

Bioinformatic analyses can be used to examine variability in genetic loci previously identified to affect the response to quaternary ammonium compounds, desiccation, or biofilm formation. Fox et al. (2011) and Casey et al. (2014) identified numerous genes in *L. monocytogenes* with altered transcription following exposure to quaternary ammonium compounds. Transposon mutagenesis studies were used to identify genes affecting desiccation response (Hingston et al., 2015), and biofilm formation (Piercey et al., 2016). Variations in amino acid sequences between strains, especially variations affecting protein properties such as hydrophobicity or catalytic sites could indicate a link to observed differences among strains' responses to desiccation, sanitizer application, or biofilm formation. In contrast, no or few differences in amino acid sequences between strains may indicate a particular gene has little impact on the phenotypic differences observed between strains.

The objectives of this research were to compare 14 *L. monocytogenes* strains isolated in Canada with respect to their ability to form biofilm formation, sensitivity to BAC and resistance to desiccation. The relationship between the presence or absence of the SSI-1 and LGI1 genetic markers and observed phenotypes of strains was tested. Using genetic sequence data, predicted

differences in selected proteins previously associated with responses to desiccation, quaternary

ammonium compounds, or biofilm formation was subsequently explored for four isolates.

2.0 Materials and methods

2.1 Bacterial strains and culture conditions

L. monocytogenes strains 08-5578, 01-7210, 08-7376, 08-6040, 06-6956, and 06-6837

were generously donated by Dr. Gilmour of the National Microbiology Laboratory, Public

Health Agency of Canada (Winnipeg, MB, Canada), and are described in Gilmour et al. (2010)

and Knabel et al. (2012). Meat and seafood processing associated strains (collected 1999- 2002

by the Canadian Food Inspection Agency, Dartmouth, NS, Canada, described by Loder, 2006)

LmG, NB1, and Lm568 (described in Kalmokoff et al. 2001) were also selected. Five urban

watershed *L. monocytogenes* isolates were collected in Nova Scotia (Canada) in 2012-2013 as

described by Stea et al. (2015), and designated 'CP' (Table 1). Routine culturing was carried out

in Tryptic Soy Broth (TSB, Oxoid, Nepean, ON, Canada), or TSB with 1% glucose (Fisher

Scientific, Whitby, ON, Canada) (TSB-glu), or on TSB with 1.5% (w/w) technical agar (Difco).

2.2 PCR based method to determine the presence of markers for the Stress Survival Islet (SSI-1), *Listeria* Genomic Island 1 (LGI1), and clonal complex 8 (CC8)

DNA was extracted using the Triton X-100 method (Liu, 2008). Briefly, a mixture of 5

mM Tris/EDTA with 1% (v/v) Triton X-100 (Sigma Aldrich) was added to overnight cultures

grown in TSB. Samples were vortexed, boiled at 95°C for 30 minutes, and cooled on ice. The

supernatant recovered after centrifuging (10 min, 10,000 x g) was then frozen (-20°C) for later

use.

7

PCR was performed to determine the presence or absence of genetic markers SSI-1, LGI1, or CC8 in the extracted DNA of each isolate. Primer sets lmo0448, representing the SSI-1 (Ryan et al., 2010), 2229, representing the CC8 group (Knabel et al., 2012), and 1886, representing the 50 kbp LGI1 island, (Knabel et al., 2012) were used in separate PCR reactions (primers described in Table 2). Using a Biometra T-Gradient thermocycler (Biometra, Goettingen, Germany), cycles included an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 40 s, annealing for 45 s, at 55°C for primer set lmo0448, or 58°C for primer sets 1886 and 2229, and extension at 72°C for 75 s. A final extension at 72°C for 5 min was implemented at the end of the last cycle. Each PCR reaction mixture consisted of 1 μl DNA (diluted from the above extraction 1/10 with DEPC water), 10 μM of each primer (0.5 μl each), 1.25 U taq DNA polymerase (0.25 μl, IDTaq[tm] Taq Polymerase kit, London, ON, Canada), 1X $MgCl_2$ free buffer (2.5 µL, 10X $MgCl_2$ free buffer, IDTaq[tm] Taq Polymerase kit), 2.5 mM $MgCl_2$ (2.5 µL, 25 mM $MgCl_2$, IDTaq[tm] Taq Polymerase kit), 10 mM dNTPs, (0.5 μl, IDTaq[tm] Taq Polymerase kit), and 17.25 µl DEPC water for a 25 µL total volume. PCR products were visualized in 2% agarose (Fisher Scientific, Oakville, ON, Canada) gels, containing 0.016% (v/v) GelRed (Biotium, Hayward, CA, USA).

2.3 Biofilm formation and desiccation survival in pre-formed biofilms exposed to 23% relative humidity (RH)

Cultures were grown in TSB-glu at 15°C for 48 h and standardized to an absorbance (450 nm, $A_{450 nm}$) of ~1.0 in TSB-glu. After diluting the standardized culture in TSB-glu, 10 μl was pipetted onto the surface of a stainless steel coupon (SS, 314, type 4 finish, 0.5 x 0.5 cm size) at an initial concentration of ~4 Log CFU/cm^2. Coupons were placed in a humidity chamber

8

(100% RH) for 48 h to form biofilm at 15°C. To desiccate the pre-formed biofilms, the coupons were then moved to a desiccation chamber, where the RH had been conditioned to 23%, by the placement of petri dishes containing saturated solutions of potassium acetate (BDH Chemicals) in the bottom of the desiccator and left to incubate for 7 days at 15°C. The RH was continuously monitored using a Gemini Tinytag View 2 datalogger (Interworld Electronics and Computer Industries, Markham, ON, Canada).

Coupons (n=3) were sampled after 0, 6, 24 and 48 h during the biofilm formation (100% RH) and after 0 (=48 h of biofilm formation), 1, 2, 3, 5 and 7 d of desiccation (23% RH). The coupons were rinsed in peptone saline (PS, 8.5 g/l sodium chloride, 1 g/l peptone, Oxoid) to remove loosely adhered cells, placed in 1 ml PS and sonicated (4 min) at room temperature (~22°C) to remove viable biofilm cells (Elmo S120H sonicating bath, Fisher Scientific) using the method of Leriche and Carpentier (1995), followed by vigorous vortexing for 60 s. Biofilm cells were enumerated after samples were serially diluted in PS, spot plated (100 μl) on Tryptone Soy Agar (TSA, Oxoid) and incubated for 48 h at 37°C. Counts were converted to Log_{10} (CFU/cm^2).

2.4 Response of strains to benzalkonium chloride

The panel of 14 strains was grown in TSB-glu at 15°C for 48 h, before standardizing the concentration as above. Cultures were then inoculated to a final concentration of ~10^6 CFU/ml in TSB-glu, or TSB-glu with benzalkonium chloride (BAC, Fisher Scientific) in microtiter plates (Costar #3370, Fisher Scientific). Plates were sealed with parafilm (Parafilm M®, Neenah WI, US), and incubated at 15°C. Concentrations of BAC ranged from 0.313 to 40 μg/ml. Controls included uninoculated media, inoculated media, and uninoculated media with BAC (0.313 μg/ml).

9

The minimum inhibitory concentration (MIC) of BAC for each strain was determined after 48 h of growth, by pipetting 75 μl of well contents into a new 96 well plate and measuring $A_{490 \, nm}$ (Biotek EL 808 Absorbance Microplate reader, Fisher Scientific).

The minimum biofilm inhibitory concentration (MBIC) of BAC for each strain was determined after 48 h and 6 days of biofilm formation at 15°C. Spent medium was discarded followed by addition of 100 μl fresh TSB-glu and 10 μl 12 mM MTT solution (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma Aldrich, Canada) to each well. Plates were incubated at 37°C for 2 h. Subsequently, 85 μl of medium was removed, 50 μl dimethyl sulfoxide (Sigma Aldrich) was added to solubilize the stain with gentle mixing. The plates were incubated for a further 10 min (37°C) before measurement of the absorbance ($A_{570 \, nm}$).

The minimum biofilm reduction concentration (MBRC) was determined as a measure of the effect of a BAC treatment on the metabolic activity of established biofilms. *Listeria* biofilms were formed (15 °C, 6 days) in wells of microtiter plates filled with TSB-glu with no BAC. After removing spent medium, aqueous BAC solutions ranging in concentrations from 0 (control) and 0.625 to 80 μg/ml were applied for 1 h at 22°C. The BAC solutions were removed, and the MTT staining protocol (above) was performed.

The following equation was used to calculate percent inhibition for MIC, MBIC, or % reduction in survival (i.e., metabolic activity) for the MBRC:

$$\% \, inhibition = \frac{(positive \, control - blank) - (test - blank)}{(positive \, control - blank)} x100$$

Each MIC, MBIC, MBRC determination was performed in triplicate, with the entire experiment repeated once (n = 6). A Student's t-test (Sigmaplot software, Systat Software Inc., San Jose, CA, USA) was used to determine the lowest BAC concentration that significantly (p<0.05) inhibited growth or biofilm formation compared to the positive control. The lowest BAC concentration that reduced the metabolic activity by ≥80% was considered to be the MBRC.

2.6 Predicted diversity in the composition of proteins associated with responses to desiccation, quaternary ammonium compounds and biofilm formation among representative phenotypes.

Four strains were used in this bioinformatics analysis: two desiccation and BAC sensitive strains (CP4 5-1, CP5 2-3) which exhibited ≥1.4 Log CFU/cm^2 loss of viable cells after desiccation for 7 days, and a 4-fold lower BAC MIC and MBIC-48 h, compared to the desiccation and BAC resistant 08-5578 strain (see Figure 1 and Table 3). Lm568 was chosen as the intermediate strain, while 08-5578 was desiccation and BAC resistant. Genomic sequences for strains Lm568 (NCBI accession number: MDRT00000000), CP4 5-1 (MDQJ00000000), CP5 2-3 (MDQI00000000) were kindly obtained from P.A. Hingston and J.C. Chen (Faculty of Land and Food Systems, University of British Columbia, BC, Canada). The genomic sequence for strain 08-5578 was obtained from the NCBI (NC_013766.2). Sequences for the genes (n=74) of interest were collected from NCBI (EGD-e, reference NC_003210.1) and from the KEGG database for LM_ 6179.

For the analysis of variability in desiccation and biofilm related proteins, eighteen genes involved in functions such as cell wall structure, internalins and transport (Hingston et al., 2015;

11

Piercey et al., 2016) were selected. For sanitizer response, genes described in Casey et al. (2014) as being upregulated in the presence of benzethonium chloride (BZT), from functional categories of peptidoglycan synthesis, fatty acid metabolism/biosynthesis, and phosphotransferase system were selected for the analysis. This included 10 proteins related to PTS system genes, 24 proteins related to peptidoglycan biosynthesis, 8 proteins related to biofilm formation, 11 related to desiccation sensitivity, 7 related to desiccation resistance, and 14 proteins related to fatty acid metabolism (n=74). It was hypothesized that the predicted amino acid sequences corresponding to genes related to biofilm formation, desiccation, or stress response would vary between strains that differed in their phenotypes.

After using BLAST to obtain sequences for the genes of interest in the four *Listeria* genomes, each gene sequence was converted to the corresponding amino acid sequence and aligned (CLC workbench, version 7.6, Qiagen Bioinformatics, Germantown, MD, USA). Differences in amino acid sequences were compared, using 08-5578 as the reference strain. Examining only the amino acid sequences ensured silent mutations would not be counted. Protein isoelectric point, aliphatic index, and number of amino acids were estimated using the CLC protein report tool (version 7, Qiagen). Annotated protein sequences in the UniProt database (UniProt, 2015) were used to determine if amino acid substitutions occurred in active sites, transmembrane domains, or other features of interest.

2.7 Statistical analysis

Total biofilm formed on stainless steel prior to desiccation (Log CFU/cm^2), and the reduction in viable counts during desiccation (ΔLog CFU/cm^2) were compared between strains at

each time point with a one way ANOVA and the Tukey-Kramer post-hoc test at significance

level of 0.05% (R based applet, Assaad et al., 2014).

        Where some data were coded as ordinal (presence/absence of genetic markers), or was

not continuous (MIC/MBIC data), a Spearman rank-order correlation coefficient test (r_s) was

used to determine correlations between factors at a significance level of 0.05% (Sigmaplot

software, Systat Inc., San Jose, CA, USA).

3.0 Results

3.1 SSI-1, LGI1, CC8 marker distribution among the panel of strains

        The SSI-1 marker was found in 10 strains including three out of five fresh water strains

(Table 1). In contrast, the LGI1 marker was only found in strains from CC8. None of the food

and fresh water isolates that originated in Atlantic Canada belonged to CC8. The five LGI1/CC8

strains also contained the SSI-1 marker.

3.2 Biofilm formation on stainless steel and desiccation survival

        Biofilm formation on SS prior to desiccation did not differ significantly ($p>0.05$) between

the strains (Table 3). All strains reached a cell density of ~7 Log CFU/cm^2 attached to the

coupons after 48 h at 15°C. When desiccated, only LGI1/CC8 strains 01-7210, 08-7376, 08-5578

exhibited no significant reduction ($p<0.05$) in viable counts, whereas all other strains displayed

varying levels of decreases in viable cell numbers (Figure 1). An intermediate desiccation

sensitivity (~0.5-1 Log CFU/cm^2 reduction by day 7) was found among four non CC8 clinical or

food processing strains (Figure 1b). In contrast, the sensitive fresh water strains underwent a ~ 1

13

Log CFU/cm^2 reduction on the third day of desiccation, and had viable counts that were 1.4-2.2 Log CFU/cm^2 lower (p<0.05) than the most desiccation resistant LGI1/CC8 strains on day 7 (Figure 1c).

3.3 Benzalkonium chloride resistance and susceptibility

For the panel of strains, the MIC for BAC ranged from 1.25-10 μg/ml (Table 3). The MBIC values after 48 h (MBIC-48 h) were similar to the MIC values. However, the BAC concentrations needed to inhibit biofilm formation over 6 days (MBIC-6 d) was 2-4 times higher than the MBIC-48 h. The BAC concentrations needed to reduce metabolic activity in a mature (6-day old) biofilm (MBRC) were 8-64 times higher than the MIC for planktonic cells. The fresh water strains typically yielded lower MIC and MBIC-48 h values as compared to clinical and food associated strains, but that trend was less clear for the MBIC-6 d values.

3.5 Spearman rank-order correlations between genetic markers, biofilm formation, and sensitivity to BAC and desiccation

This non-parametric statistical analysis showed that LGI1/CC8 strains correlated negatively with desiccation sensitivity (r_s=-0.647) and positively with increasing MIC, MBIC-48 h and MBIC-6 d values (r_s=0.556-0.748, Table 4). This meant that these strains ranked among the more desiccation resistant and required higher concentrations of BAC to inhibit planktonic and biofilm growth as well as sessile cells in mature biofilms. Ranking of the strains' biofilm formation after 48 h on SS positively correlated (r_s=0.549-0.559) with MIC, MBIC-48 h and MBIC 6 d values while the correlation with desiccation sensitivity was negative (r_s=-0.605).

The MIC values positively correlated with MBIC-48 h and MBIC-6 d, and the latter two factors also positively correlated with each other (p<0.05). This meant that strains, which were more sensitive to BAC as planktonic cells, also ranked as being more sensitive to BAC during formation of biofilm (Table 3).

3.5 Bioinformatics analysis of selected genes

For four strains, which represented desiccation and BAC sensitive, intermediate, and resistant phenotypes in this study, the predicted amino acid sequences of proteins previously linked to biofilm formation and susceptibility to desiccation and quaternary ammonium compounds were analyzed. Nucleotide changes and corresponding amino acid changes were found in 5 PTS proteins, 11 peptidoglycan proteins, 7 biofilm genes, 5 desiccation tolerance related proteins, 9 desiccation sensitivity related proteins, and 7 fatty acid metabolism proteins ($n_{alterations} = 44$). (See supplemental tables S1-S6).

Alignment in the UniProt database showed amino acid changes in protein regions predicted to affect function for 4 peptidoglycan proteins, 6 biofilm related proteins, 4 desiccation tolerance related proteins, and 3 desiccation sensitivity related proteins ($n_{functions} = 17$). Of the 17 genes with amino acid changes in regions predicted to alter function, a large amount of annotation information was available for 10 proteins as summarized in Table 5. One protein was not found in the UniProt database and not all proteins had annotations with respect to features of interest.

Several differences in amino acids between strains 08-5578 and Lm568, CP4 5-1, or CP5 2-3, were found in signal peptide regions or transmembrane domains among peptidoglycan and biofilm genes. Fatty acid metabolism genes other than *lmo1370* (butyrate kinase) did not display

amino acid differences in protein regions predicted to affect function. With the exception of *lmo0319* (phospho-beta-glucosidase), none of the phosphotransferase system (PTS) genes differed by more than two amino acids among the strains.

The sequence of 08-5578 (LGI1/CC8 desiccation and BAC resistant phenotype) for lmo1080 (teichoic acid biosynthesis) differed by one amino acid in the protein's glycosyltransferase domain, while the consensus sequence was shared by the other three strains. Only Lm568 (intermediate phenotype) differed from the other strains in lmo1083 (dTDP-D-glucose 4,6-dehydratase), with one amino acid difference in a dehydratase domain. Fresh water strains CP4 5-1, and CP5 2-3, which represented the desiccation and BAC sensitive phenotype, exhibited the same amino acid variation in a transmembrane domain in lmo0973 (D-alanine esterification of lipoteichoic acid). These strains also differed in the composition of the peptidoglycan related lmo2558 (autolysin amidase).

Biofilm related internalins (lmo0433 [InlA], lmo0434 [InlB], lmo0263 [InlH]) demonstrated the highest number of amino acid differences among the strains. Internalin H (lmo0263) in the desiccation and BAC sensitive strain CP5 2-3, showed many differences in the signal peptide region compared to the other strains. Also, several amino acid variations in lmo0433 and lmo0434 were found in the desiccation and BAC resistant 08-5578 strain, relative to the other three strains, which harboured similar *inlA* and *inlB* sequences. These differences in amino acid composition for internalin genes were mapped to signal peptide regions, tandem repeats, or leucine rich repeat regions. The resistant 08-5578 strain also contained differences in the amino acid sequence within periplasmic and transmembrane domains of biofilm associated lmo1224 (ABC-type transporter).

16

Amino acid variations were located in the transmembrane domain of lmo0771 (hypothetical protein, desiccation tolerance) in the intermediate strain Lm568. The sensitive strain CP5 2-3 contained an amino acid variation in the epimerase domain of lmo1744 (hypothetical protein, desiccation tolerance).

As previously described, strain 08-5578 had a frameshift mutation leading to a premature stop codon (PMSC) in *lmo1370* (Gilmour et al., 2010), which was not observed in the other three strains. No other examined genes (including internalins) were found to harbour PMSCs.

4.0 Discussion

Five *Listeria monocytogenes* strains were found to have all the genetic markers evaluated (LGI1, CC8 and SSI-1), and to be significantly (p<0.05) more desiccation and BAC resistant relative to the other nine strains examined in the study.   Knabel et al. (2012) found LGI1 in 90% of all Canadian CC8 strains tested. However, not all CC8 strains contain the LGI1 marker, whose prevalence appears to vary geographically (Althaus et al., 2014).

An additional five strains also possessed the SSI-1 marker. This finding concurs with the original study by Ryan et al. (2010) where half of all isolates tested contained the marker, and also the high SSI-1 incidence found in CC8 clinical isolates in a Swiss study (Althaus et al., 2014). However, in the current study, no significant (p>0.05) correlations were found for the presence of SSI-1 and desiccation survival, biofilm formation, or resistance to BAC for either planktonic or biofilm cells. Ebner et al. (2015) reported finding no role of SSI-1 in the ability of strains to form biofilm. Taken together, SSI-1 appears to be widespread, but does not appear to be a major determinant of survival during exposure to the food processing stresses tested here.

Moreover, Arguedas-Villa et al. (2014) found *L. monocytogenes* strains isolated in Switzerland and Canada that harboured SSI-1 did not exhibit enhanced cold growth.

LGI1 positive *L. monocytogenes* strains had significantly (p<0.05) greater resistance to BAC both as planktonic and sessile biofilm cells as compared to LGI1 negative *L. monocytogenes* strains. Ziegler (2011) similarly found that LGI1 isolates had higher MIC values for BAC and a related sanitizer BZT. In a recent study, Kovacevic et al. (2016) reported the higher BAC resistance occurred due to LGI1's *emrE* efflux activity. Aside from the efflux genes, LGI1 also contained genes that code for type II and type IV secretion systems, a peptidoglycan hydrolase with a possible positive role in biofilm formation (Mercier et al., 2002), and an adhesin, which in combination, could lead to altered biofilm formation (Gilmour et al., 2010). Interestingly, exposure to BZT was reported to increase expression of peptidoglycan related genes in a persistent *L. monocytogenes* strain (Fox et al., 2011). Therefore, it may be that biofilm formed by LGI1 positive *L. monocytogenes* strains conveyed resistance to BAC as well as desiccation. Differences in biofilm maturity have previously been shown to influence desiccation survival even if the initial levels of viable cells exposed to desiccation were similar (Hingston et al., 2013). It is important to note that due to the co-occurrence of SSI-1 with LGI1 in the studied isolates, it is possible the SSI-1 exerted an effect when combined with LGI1. Further studies should be focussed to determine the precise function of LGI1 in the presence and absence of SSI-1 in order to elucidate their roles in the growth and survival of *L. monocytogenes* in food processing plants.

The non-parametric Spearman rank correlation revealed no significant (p > 0.05) link between biofilm formation and being a LGI1/CC8 strain. However, BAC resistance and desiccation resistance correlated significantly (p<0.05) with the LGI1/CC8 isolates. Biofilm

formation also correlated (p<0.05) with resistance to desiccation and BAC. Nakamura et al. (2013) observed in a crystal violet assay higher levels of biofilm for a persistent (and BAC resistant) *L. monocytogenes* strain relative to a transient strain, indicating this persistent strain produced greater amounts of EPS as visualized by the CV stain. However, their results showed no significant differences in the viable cell counts in the biofilms produced by these strains.

The ability of BAC to affect cells in mature biofilms (MBRC) after 6 days did not vary greatly among strains, which may be due to the tested concentration range or their mature biofilms being similar (Table 3). Harvey et al. (2007) and Wang et al. (2015) also reported that initial variations in biofilm formation between strains diminished with sufficient incubation time.

Fresh water isolates tended to be more sensitive to BAC compared to clinical and food associated strains, and they were also more susceptible to desiccation. Water associated *L. monocytogenes* strains are known to enter the food chain (Weller et al., 2015), and are often similar to serotypes or pulsotypes known to cause illness (Lyautey et al., 2007; Stea et al., 2015). However, if strains with phenotypes similar to this study's fresh water isolates entered food processing facilities, they should be less difficult to manage from a sanitation standpoint. In contrast, the finding of the enhanced survival of food and clinical LGI1/CC8 strains when exposed to stresses commonly occurring in the food industry may help to explain the prior research that found a long term predominance of this group in clinical cases in Canada over a 30-year period (Knabel et al., 2012).

It was hypothesized that the predicted amino acid sequences corresponding to genes related to biofilm formation, desiccation, or stress response would vary between strains that differed in their phenotypes. The bioinformatics results suggest that the PTS and many fatty acid metabolism genes examined may be more conserved than genes in other functional categories.

Among the strains examined, variability in amino acid sequences, and ensuing variability in protein domains which may affect function, was observed more often in genes relating to peptidoglycan biosynthesis, biofilm formation, and desiccation.

Interestingly, *lmo1080* has been found to be related to both biofilm formation (Piercey et al., 2016) and teichoic acid biosynthesis (Eugster et al., 2015; Vergara-Irigaray et al., 2008). Moreover, *lmo1080* and *lmo1083* are transcribed in the same operon, and the latter also appears to impact biofilm formation (Ouyang, 2010). Further, both genes were found to exhibit altered transcription when exposed to BZT (Casey et al., 2014). In this study, the observed variations in the predicted amino acid sequences of lmo1080 of 08-5578 coincided with increased BAC and desiccation tolerance while observed changes in lmo1083 of CP4 5-1 occurred together with reduced BAC and desiccation tolerance.

The two sensitive fresh water strains had amino acid differences in predicted functional domains of lmo0973 and lmo2558. *Lmo0973 (DltB)* is peptidoglycan related, but is also known to affect biofilm formation on polystyrene and stainless steel (Alonso et al., 2014; Piercey et al., 2016). In addition, a disruption in this gene was demonstrated to impact BAC sensitivity following biofilm formation (Piercey et al., 2016). Altered biofilm formation is also known to occur when cell wall related autolysin amidase is disrupted (*lmo2558*) (Piercey et al., 2016). This gene is also known to affect adhesion to eukaryotic cells (Milohanic et al., 2001), yet its precise role in biofilm formation, BAC tolerance, and desiccation survival remains unclear.

For the biofilm related transport gene *lmo1224*, the predicted amino acid sequence for strain 08-5578 differed from the other examined strains in a predicted periplasmic domain. This domain has structural similarity to the AcrB multidrug efflux transporter (Bateman et al., 2004). It seems possible that variation may influence the efflux of BAC, as the periplasmic domain of

AcrB is known to influence ligand binding of sanitizers and MICs in *Escherichia coli* (Edward et al., 2005). The predicted amino acid variation may help to explain the increased resistance of strain 08-5578 to BAC in the current study.

Internalin proteins are thought to affect biofilm in *Listeria monocytogenes* as well as its adhesion, virulence, internalization into eukaryotic cells and survival in different environmental niches (Cabanes et al., 2002; Franciosa et al., 2009; Orsi et al., 2010; Piercey et al., 2016). Therefore, it was not surprising to see amino acid variations in internalin sequences among strains that varied in their response to BAC or desiccation. Lmo0263 was found to have numerous amino acid variations in functional domains in the sensitive strain CP5 2-3, while lmo0433 and lmo0434 were predicted to contain many amino acid variations in functional domains, in the resistant strain 08-5578.

For the desiccation tolerance related to *lmo0771* (Hingston et al., 2015) transcription levels of that gene are known to increase in cells exposed to UV treatment (Uesugi et al., 2016), low pH, and low water activity (Zhang et al., 2010). Amino acid variations in this gene co-occurring with the intermediate resistant strain Lm568, may be consistent with the gene's role in stress survival. Interruption of *lmo1744* conferred desiccation tolerance (Hingston et al., 2015), which is interesting considering the response regulator affects the *dltABCD* operon (Mandin et al., 2005), thereby probably impacting *dltA*, *dltB,* and *dltD* and their influence on biofilm formation (Alonso et al., 2014; Ouyang et al., 2012; Piercey et al., 2016). Amino acid variations in lmo1744 were found in one of the sensitive fresh water strains and could potentially have affected both biofilm formation and desiccation resistance.

The frameshift mutation and truncation of *lmo1370* (butyrate kinase*)* in strain 08-5578 corresponding with heightened desiccation resistance is consistent with prior research that found

interruption of *lmo1370* increased thermal tolerance (Ells et al., 2009), desiccation resistance and osmotolerance in *L. monocytogenes* (Hingston et al., 2015). Strain 08-7376 has the same frameshift in *lmo1370* as was found in strain 08-5578 (Gilmour et al., 2010), and in this study exhibited a similar increased resistance to desiccation as 08-5578. It is notable that strain 08-6040 harboured the SSI-1 and LGI1 genetic markers as well as the frameshift mutation in *lmo1370*, yet it only showed moderate ability to survive desiccation stress, while it was relatively resistant to BAC.

It appears numerous genetic factors have an effect on the variations in biofilm formation, desiccation and BAC resistance observed among the panel of *L. monocytogenes* strains in this study, including the presence or absence of the LGI1/CC8 marker. It should be noted that all functional categories examined in the bioinformatics analysis included genes which demonstrated no differences in predicted amino acid sequences among the strains. Therefore, the different phenotypes are likely the net result of complex diversity of amino differences in several proteins.

In conclusion, LGI1/CC8 strains may pose a serious challenge to sanitation efforts due to their enhanced survival when exposed to desiccation and a commonly used sanitizer. Circulation of such resistant strains in the food industry could severely affect food safety. Bioinformatic analysis of selected biofilm, desiccation and BAC related genes uncovered some predicted amino acid sequence differences where variability may have affected the response to the studied environment and sanitation stresses. Further studies are needed to determine if such genes may serve as targets for detection of strains with elevated resistance to stresses encountered in the food processing environment.

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23

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24

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Table 1. *Listeria monocytogenes* strain origin, and presence or absence of genetic markers, SSI-1 (Stress Survival Islet 1, Ryan et al. 2010), the 50 kbp LGI1 (Listeria Genomic Island 1, described in Gilmour et al., 2010), and CC8 marker (Clonal Complex 8, described in Knabel et al., 2012).

Listeria monocytogenes strain	Origin	Serotype	Serogroup [a]	SSI-1	LGI1	CC8	Reference
01-7210	Liverwurst Sausage	1/2a	IIa	+	+	+	Knabel et al., 2012
08-7376	Environment-Food Processing	1/2a	IIa	+	+	+	Gilmour et al., 2010
08-5578	Blood- 2008 RTE meat outbreak	1/2a	IIa	+	+	+	Knabel et al., 2012
NB1	Smoked Salmon	1/2a	IIa	+	-	-	Loder, 2006
06-6837	Blood	1/2a	IIa	+	+	+	Knabel et al. 2012
06-6956	Blood	1/2a	IIa	-	-	-	Knabel et al., 2012
Lm568	Food processing environment	1/2a	IIa	-	-	-	Kalmokoff et al., 2001
08-6040	Food – 2008 RTE meat outbreak	1/2a	IIa	+	+	+	Gilmour et al., 2010
LmG	Food processing environment	1/2b	IIb	+	-	-	Loder, 2006
CP5 2-3	Fresh water		IIa	+	-	-	Stea et al., 2015
CP5 2-2	Fresh water		IIa	+	-	-	Stea et al., 2015
CP5 2-1	Fresh water		IIa	+	-	-	Stea et al., 2015
CP4 5-1	Fresh water		IIa	-	-	-	Stea et al., 2015
CP4 5-2	Fresh water		IIa	-	-	-	Stea et al., 2015

[a] Serogroup IIa is most often serotype1/2a or 3a

Table 2. Primers used in CC8, LGI1, and SSI-1 marker detection.

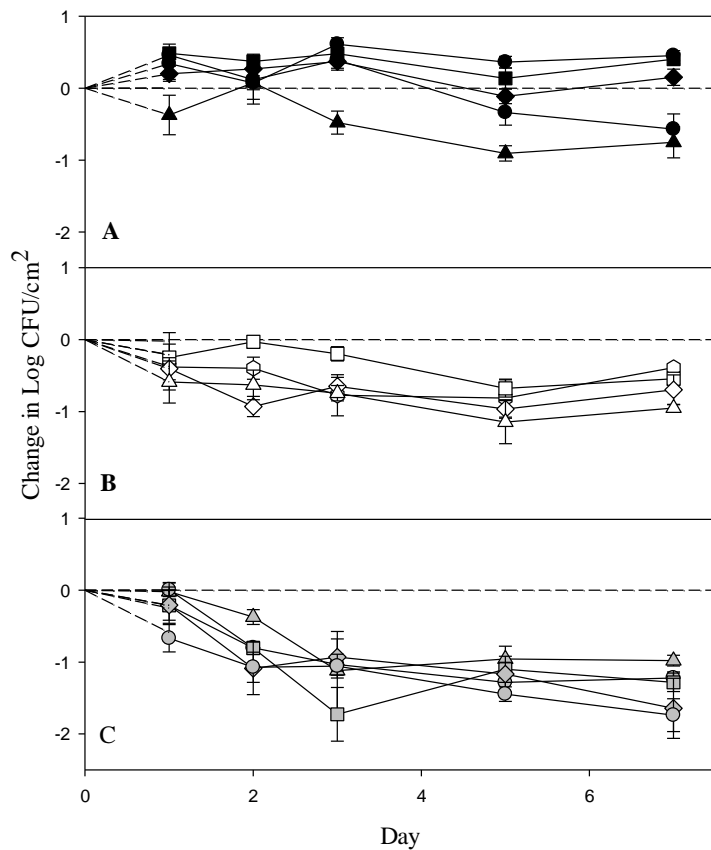| Marker	Primer Name	5' to 3' Sequence	Reference
CC8	2229F	TTGTTGAAGGAAGAGGTGGTC	Knabel et al., 2012
	2229R	TCTTTTCGGCTCATTTTCGT	
LGI1	1886F	TAACGACAACTATGCCAACG	Knabel et al., 2012
	1886R	CGACAGTTTGTATTCCACCA	
SSI-1	lmo0448F	CTTGCAGCAAGCTTCATC	Ryan et al., 2010
	lmo0448R	CATAAGAATCCACATAGTAG	

Figure 1. Change in Log CFU/cm^2 of *L. monocytogenes* during desiccation (23% RH, 15°C) after prior biofilm formation (48 h, 15 °C, 100% RH, n = 3). (A) LGI1/CC8 isolates, black symbols; (B) non CC8 clinical and food processing strains, open symbols; (C), fresh water isolates, grey symbols;. Dashed lines represent the period where relative humidity changed from 100% to 23%.

Table 3. Concentrations of benzalkonium chloride (µg/ml) required to inhibit planktonic growth (MIC), inhibit biofilm formation (MBIC) over 48 h or 6 days, or reduce ≥80% of the metabolic activity in pre-formed 6-day old biofilm (MBRC) at 15°C.

Strain	MIC 48 h	MBIC-48 h	MBIC-6 d	MBRC 6 d	LGI1-CC8 strain	Biofilm formed on SS coupons (Log CFU/cm^2)^a
01-7210	10	5	10	80	+	7.22±0.07 a
08-7376	10	5	10	>80^b	+	7.35±0.10 a
08-5578	5	5	10	80	+	7.42±0.10 a
NB1	10	10	10	80	-	7.39±0.09 a
06-6837	5	5	10	>80	+	7.12±0.23 a
06-6956	2.5	1.25	10	80	-	7.33±0.11 a
Lm568	2.5	1.25	5	80	-	7.30±0.19 a
08-6040	5	10	10	80	+	7.34 ±0.07 a
LmG	2.5	1.25	5	80	-	7.16 ±0.09 a
CP 5 2-3	1.25	1.25	5	80	-	7.17 ±0.13 a
CP 5 2-2	1.25	1.25	10	80	-	7.30 ±0.10 a
CP 5 2-1	1.25	1.25	5	80	-	7.20 ±0.13 a
CP 4 5-1	1.25	1.25	5	80	-	7.06 ±0.14 a
CP 4 5-2	1.25	1.25	10	80	-	7.18 ±0.04 a

^{a}Different letters within the same column indicate significant differences by the Tukey Test (p<0.05).
^{b} The highest BAC concentration used in this assay (80 µg/ml) was unable to reduce ≥80% of the metabolic activity in pre-formed biofilms made by this strain.

Table 4. Spearman rank-order correlation coefficients (r_s) between the LGI1/CC8 and SSI-1 markers, biofilm formed after 48 h, desiccation susceptibility, MIC, MBIC-48 h and MBIC-6 d. Significant ($p \le 0.05$) r_s-values are shown, while non-significant r_s-values are indicated by NS.

	SSI-1	LGI-1/CC8	MIC 48 h	MBIC-48 h	MBIC-6 d	Desiccation sensitivity [a]
MIC 48 h	NS	0.710	-	-	-	-
MBIC-48 h	NS	0.748	0.861	-	-	-
MBIC-6 d	NS	0.556	0.556	0.624	-	-
Desiccation sensitivity[a]	NS	-0.647	-0.909	-0.667	NS	-
Biofilm formed on SS (48 h)[b]	NS	NS	0.559	0.549	0.555	-0.605

[a] Based on the reduction in viable counts (Δ Log CFU/cm^2) at 7 days.
[b] Log CFU/cm^2.

Table 5. Apparent phenotypic change and variations in predicted amino acid composition affecting selected protein's structural/active domains in four *L. monocytogenes* isolates with sensitive (CP4 5-1, CP5 2-3), intermediate (Lm568) and resistant (08 5578) phenotypes in relation to benzalkonium chloride (BAC) and desiccation tolerance.

Gene Description (uniprot database designation)	Reference(s) where gene was related to biofilm formation or sensitivity to BAC or desiccation	Amino acid differences in predicted structural/active regions of protein[a]	Apparent phenotypic change
D-alanine esterification of lipoteichoic acid and wall teichoic acid *(dltB)*, *lmo0973* (Q7AP77)	Piercey et al., 2016	Transmembrane domain	Reduced BAC/desiccation tolerance
Teichoic acid biosynthesis protein *GgaB*, *lmo1080* (Q8Y838)	Casey et al., 2014; Piercey et al., 2016	Glycosyltransferase domain	Increased BAC/desiccation tolerance
dTDP-D-glucose 4,6-dehydratase, *lmo1083*(Q8Y835)	Casey et al., 2014, Ouyang et al., 2012	GDP-mannose 4,6 dehydratase domain	Reduced BAC/desiccation tolerance
Autolysin amidase (*ami*), *lmo2558* (Q8Y496)	Piercey et al., 2016	Chain domain	Reduced BAC/desiccation tolerance
Internalin H (*inlH*), *lmo0263* (Q7AP87)	Piercey et al., 2016	Signal peptide region	Increased BAC/desiccation tolerance
Internalin A (*inlA*), *lmo0433* (P0DJM0)	Piercey et al., 2016	Signal peptide, leucine rich repeat regions, tandem repeat region	Increased BAC/desiccation tolerance
Internalin B (*inlB*), *lmo0434* (P25147)	Piercey et al., 2016	Signal peptide, leucine rich repeat regions	Increased BAC/desiccation tolerance
ABC-type transporter, *lmo1224* (Q8Y7P9)	Piercey et al., 2016	Periplasmic and transmembrane domains	Increased BAC/desiccation tolerance
Hypothetical protein, *lmo0771* (Q8Y8X1)	Hingston et al., 2015	Transmembrane domain	Moderately increased BAC/desiccation tolerance
Hypothetical protein, *lmo1744* (Q8Y6E3)	Hingston et al., 2015	Epimerase domain	Reduced BAC/desiccation tolerance
Butyrate kinase, (*buk*) *lmo1370* (Q8Y7B6)	Hingston et al., 2015, Gilmour et al., 2010	Frameshift /truncation in chain region	Increased desiccation tolerance

[a]For detailed information describing the 74 genes screened, and precise locations of amino acid differences, see Supplementary Tables S1-S6.

Research Highlights:

- *L. monocytogenes* CC8 strains from Canada contained both the SSI-1 and LGI1 markers

- The LGI1/CC8 strains exhibited increased resistance to the BAC sanitizer

- Aquatic *L. monocytogenes* were more susceptible to desiccation than LGI1/CC8 strains

- Varying BAC/desiccation resistance coincided with differences in membrane/biofilm

  genes