Pre-growth conditions and strain diversity affect nisin treatment efficacy against *Listeria monocytogenes* on cold-smoked salmon

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ABSTRACT

Listeria monocytogenes is a human pathogen that is commonly found in environments associated with cold-smoked salmon. Nisin is a natural antimicrobial that can be used as a food preservative. While nisin is active against a number of Gram-positive bacteria, including *L. monocytogenes*, environmental stresses encountered in cold-smoked salmon processing facilities might affect *L. monocytogenes*' nisin susceptibility. The objective of this study was to investigate the effect of seafood-relevant pre-growth conditions and *L. monocytogenes* strain diversity on nisin treatment efficacy on cold-smoked salmon. Six *L. monocytogenes* strains representing serotypes most commonly associated with cold-smoked salmon (1/2a, 1/2b, and 4b) were initially pre-grown under a number of seafood-relevant conditions and challenged with nisin in growth media modified to represent the characteristics of cold-smoked salmon. The pre-growth conditions with the lowest mean log reduction due to nisin and the highest strain-to-strain variability were selected for experiments on cold-smoked salmon; these included: (i) 4.65% w.p. NaCl ("NaCl"); (ii) pH=6.1 ("pH"); (iii) 0.5 μg/ml benzalkonium chloride ("Quat"); and a control ("BHI") . Cold-smoked salmon slices with or without nisin were inoculated with *L. monocytogenes* pre-grown in one of the conditions above, vacuum-packed, and incubated at 7°C. *L. monocytogenes* were enumerated on days 1, 15 and 30. A linear mixed effects model was constructed to investigate the effect of pre-growth condition, day in storage, serotype, source of isolation as well as their interactions on nisin efficacy against *L. monocytogenes*. Compared to pre-growth in "BHI", significant reduction ($P < 0.05$) in nisin efficacy was induced by pre-growth in "pH" and "Quat" on both days 15 and 30, and by pre-growth in

"NaCl" on day 30, indicating a time-dependent cross-protection effect. Additionally, an effect of *L. monocytogenes*' serotype on the cross-protection to nisin was observed; pre-growth in "pH" significantly reduced nisin efficacy against serotype 1/2a and 4b strains, but not against 1/2b strains. In conclusion, pre-exposure to mildly acidic environment, high salt content, and sublethal concentrations of quaternary ammonium compounds, is likely to provide cross-protection against a subsequent nisin treatment of *L. monocytogenes* on cold-smoked salmon. Therefore, challenge studies that use pre-growth in "BHI", as well as more susceptible *L. monocytogenes* strains, may overestimate the efficacy of nisin as a control strategy for cold-smoked salmon.

Keywords: pH, salt, Quaternary ammonium compound, Cross-protection, RTE seafood.

1. INTRODUCTION

Listeria monocytogenes causes a potentially severe foodborne disease called listeriosis that is responsible for approximately 260 deaths annually in the US, making it the 3rd leading cause of death from foodborne illnesses (CDC, 2017; Scallan et al., 2011). Individuals that are particularly susceptible to listeriosis include pregnant women and their newborns, elderly people, and immuno-compromised individuals (CDC, 2017). In addition, *L. monocytogenes* is a particularly problematic food-borne pathogen because it is able to grow in a wide range of stresses including high salt content and low temperature (Doyle et al., 2019). While this pathogen typically contaminates food at low levels that are unlikely to cause human disease, it can grow to levels that represent considerable risk of causing listeriosis during refrigerated storage of many ready-to-eat

(RTE) foods (Gombas et al., 2003). Consequently, U.S. regulatory policy specifies zero-tolerance (i.e., absence in 25 g) for *L. monocytogenes* in RTE foods (Archer, 2018), including cold-smoked salmon.

Among all recent recalls due to contamination of *L. monocytogenes* for foods regulated by the U.S. Food and Drug Administration (FDA) (2017 - 2020), seafood associated products account for approximately 8.77% (FDA, 2020). Compared to frozen and processed seafood, control of *L. monocytogenes* in cold-smoked fish products, such as cold-smoked salmon, remains particularly challenging (Norton et al., 2001; Sabanadesan et al., 2000). This could be due in part to the fact that (i) raw salmon is often naturally contaminated with *L. monocytogenes* (Di Ciccio et al., 2012), (ii) the temperature used for cold smoking (below 30°C) is not adequate for *L. monocytogenes* inactivation (Cornu et al., 2006; Eklund et al., 1995), (iii) the intrinsic characteristics of cold-smoked salmon can support growth of *L. monocytogenes* (Guyer and Jemmi, 1991), and (iv) cold-smoked salmon is stored at refrigeration temperature (which allows for growth of *Listeria*) for long periods of time (up to 60 days) (Kang et al., 2012). Therefore, the development of novel and the optimization of current control strategies for *L. monocytogenes* in cold-smoked salmon is of crucial importance for this industry.

Nisin, an FDA-approved natural antimicrobial produced by *Lactococcus lactis*, is effective against a wide range of Gram-positive bacteria, including *L. monocytogenes* (Delves-Broughton et al., 1996). Application of nisin has been reported to cause a significant reduction of *L. monocytogenes* on cold-smoked salmon (Neetoo, 2008; Nilsson et al., 1997; Ye et al., 2008). The mechanism of action of nisin involves binding of Lipid II and formation of pores in the cell membrane (Bierbaum and Sahl, 2009;

92 Hasper et al., 2006; Nilsson et al., 1997), inducing cell lysis. Different mechanisms have
93 been reported to confer resistance to nisin, including the d-alanylation of wall teichoic
94 acids (WTA) by the products of the *dltA* operon (Reichmann et al., 2013), which is
95 regulated by the VirRS two component system (Mandin et al., 2005). While the cell
96 envelope of Gram-positive bacteria has a net negative charge, the incorporation, through
97 d-alanylation, of d-alanine into WTA contributes to a change in charge that presumably
98 prevents action of cationic antimicrobial peptides such as nisin (Reichmann et al., 2013).
99 Changes in cell membrane composition, such as those causing a reduced membrane
100 fluidity, can also affect nisin susceptibility (Ming and Daeschel, 1993, 1995). As many of
101 these mechanisms are differentially expressed under different environments, it is likely
102 that growth conditions affect nisin susceptibility.
103 It has long been recognized that different strains can vary considerably in their
104 intrinsic resistance to different types of stress, highlighting the importance of accounting
105 for strain diversity to assure a broad efficiency of control strategies. Different subtyping
106 methods have grouped *L. monocytogenes* into four evolutionary distinct lineages (I, II, II
107 and IV). Most isolates, however, are grouped within lineage I or II (Orsi et al., 2011).
108 Lineage I and II include serotypes 1/2b and 4b, and serotype 1/2a, respectively, the
109 serotypes more commonly associated with human clinical cases (Orsi et al., 2011).
110 Several studies have shown that different strains of *L. monocytogenes* differ in their
111 ability to survive under stress conditions (Bergholz et al., 2010; Cheng et al., 2015; De
112 Jesús and Whiting, 2003; Lianou et al., 2006). For example, it has been shown that at
113 37°C, *L. monocytogenes* lineage I and III strains show increased salt resistance as
114 compared to lineage II strains, while at 7°C, most strains representative of different *L.*

115 *monocytogenes* lineages (I to IV) react similarly to salt stress (Bergholz et al., 2010).
116 Additionally, lineage III strains have been shown to be more effectively inactivated by
117 heat treatment, however, considerable strain-to-strain variation within lineages has also
118 been observed in all lineages tested (I to III) (De Jesús and Whiting, 2003). Taken
119 together, these findings illustrate the importance of using strains representing different
120 lineages and serotypes in studies on *L. monocytogenes* stress survival as well as in
121 validation studies for control strategies.
122 More recently, the effect of pre-growth condition has been identified as an
123 additional important factor to consider for the efficiency of control strategies against
124 foodborne pathogens (Harrand et al., 2019). Since cell envelope composition and
125 structure, as well as expression of cell envelope related genes, can be severely affected by
126 environmental and stress conditions, one could hypothesize that the efficiency of cell-
127 wall acting control strategies against *L. monocytogenes* also might be affected by the
128 environmental condition a given strain has been or is exposed to. In fact, multiple studies
129 have shown that the conditions under which cells are pre-grown have a considerable
130 impact on the ability of *L. monocytogenes* to survive a subsequent stress. For example,
131 pre-exposure to salt and potassium lactate appears to increase *L. monocytogenes*
132 resistance to nisin in broth (Bergholz et al., 2013; Kang et al., 2015). Short- and long-
133 term adaptation to salt stress has been found to induce cross-protection against other
134 stresses, including oxidative stress (Bergholz et al., 2012). Another study has shown that
135 acid stress and desiccation increase UV resistance in *L. monocytogenes* and other
136 pathogens (Gabriel, 2015). Taken together, all of these studies highlight the underlying

importance of considering the growing environment of *L. monocytogenes* (e.g. in the processing plant) for challenge and validation studies of different control strategies.

Understanding how pre-exposure to seafood-relevant conditions, as well as strain diversity, affect *L. monocytogenes* prior to exposure to a control strategy is essential to allow for development of new and improved interventions for smoked seafood products and to correctly assess the efficacy of already-existing interventions. In this study, we sought to explore the effect of pre-growth condition and strain diversity on nisin treatment efficacy against *L. monocytogenes* present on cold-smoked salmon.

2. MATERIALS AND METHODS

2.1 Assembly and characterization of seafood-relevant strain collection

A total of six strains were selected from a collection of more than 13,432 *L. monocytogenes* isolates in food microbe tracker (http://www.foodmicrobetracker.com/login/loginagain.aspx). The criteria used to select these strains were (i) to represent the serotypes (i.e., 1/2b, 1/2a, and 4b), ribotypes (e.g., DUP-1039, DUP-1062), and lineages (i.e., I and II) commonly associated with cold-smoked salmon production (Dauphin et al., 2001; Di Ciccio et al., 2012; Hoffman et al., 2003; Vongkamjan et al., 2013) and (ii) to include strains that were isolated from both smoked fish finished product as well as processing facility environments (Table 1). All strains in the collection were maintained at -80°C in Brain Heart Infusion (BHI) broth with 15% (v/v) glycerol.

2.1.1 Whole genome sequencing (WGS) analysis

All six strains were characterized by WGS using an Illumina Miseq instrument (New York State Veterinary Diagnostic Laboratory, Cornell University). Raw sequencing reads were trimmed based on the quality of the bases sequenced and adaptors were clipped off using Trimmomatic version 0.36 (Bolger et la., 2014). The overall quality of the remaining raw sequencing reads was assessed using FASTQC version 3.10.1 (Andrews, 2010). Trimmed reads were *de novo* assembled using SPAdes version 3.10.1 (Nurk et al., 2013). Contigs with less than 200 bases were screened out from the assemblies to result in the final assemblies (draft genomes). The quality of the final assemblies was verified using QUAST version 4.0 (Mikheenko et al., 2016). The average coverage was estimated by mapping the trimmed raw reads back to the corresponding final assembly using BBMap version 37.50 (Bushnell, 2014) followed by Samtools version 1.6 (Li et al., 2009). Subtyping was conducted for all strains using the core genome multi locus sequence typing (cgMLST) scheme available at the BIGSdb-*Lm* database (Moura et al., 2017) with 283 reference genomes downloaded from the NCBI Refseq database. A dissimilarity matrix was then used to perform a hierarchical cluster analysis using the complete linkage method implemented in the function "hclust" from the stats (v 3.6.2) package in R Statistical Programming Environment (R core team, 2018). Raw sequence data for all six strains have been deposited in the NCBI Sequence Read Archive (SRA) with the accession numbers: SRR6795895 (FSL L3-0051), SRR6796400 (FSL L4-0060), SRR6796397 (FSL F2-0310), SRR6796398 (FSL N1-0061), SRR6796395 (FSL F2-0237), and SRR6795875 (FSL L4-0396).

2.1.2 *In silico* serotyping and genotypic characterization

183 Lineage and serotype information of the six strains was confirmed using two different
184 methods: (i) cgMLST clustering with reference strains and (ii) *in silico* serotyping.
185 Initially, the lineage and serotype of selected reference strains clustered with each of the
186 six strains (based on our phylogenetic analysis) were visually inspected on the NCBI
187 website. The serotype of the six strains was further confirmed using an *in silico*
188 serotyping method (Doumith et al., 2004; Huang et al., 2011). Briefly, the nucleotide
189 sequences for five genes (*orf2819*, *orf2110*, *lmo1118*, *lmo0737*, and *prs*) were
190 downloaded from the BIGSdb-*Lm* database (Moura et al., 2017) along with the *L.*
191 *monocytogenes* PCR serogroup profile. BLASTN was then used to identify 100%
192 matches between the database sequences and our six final assemblies. An *in silico*
193 serotype was then assigned to the each of the isolates based on their five-gene profile.
194 To assess the potential ability of the six strains to respond to various stress
195 conditions, we detected the presence and integrity of selected genes that are critical for
196 general stress response (*rsbV, sigB*), nisin resistance (*virR, virS, dltA and mprF*), and
197 virulence (*inlA, prfA*). For each of these genes, the protein sequence was extracted from
198 the 10403S genome annotation and used to create a database; BLASTX was then used to
199 search through the final assembly of each of the strains for the presence of the gene (with
200 a threshold of 90% identity and 90% coverage). Potential premature stop codons were
201 identified by visual examination of the output files from BLASTX. In addition, the
202 presence of selected genes involved in quaternary ammonium compound tolerance
203 (*bcrABC, emrE, emrC, qacH, qacA, and mdrL*) was assessed. Nucleotide sequences were
204 downloaded from the BIGSdb-*lm* database (Moura et al., 2017) and used to search for the

205 presence of the respective genes (with a threshold of 70% identity and 70% coverage) in
206 the genomes of the six strains used in this study.
207
208 **2.1.3 Minimum inhibitory concentration (MIC) assay**
209 A broth microdilution method (Wiegand et al., 2008), modified according to Van Tassell
210 et al. (2015), was used to measure the MIC of nisin (Nisaplin [containing 2.5% nisin;
211 DuPont, Wilmington, DE]) for all six *L. monocytogenes* strains used in this study. Briefly,
212 two-fold serial dilutions of nisin in BHI broth were prepared in 96-well microtiter plates,
213 starting with 0.125 mg/ml. Wells were inoculated with each of the strains at a final
214 concentration of 10^5 CFU/ml. The inoculated plates were incubated at 37°C for 24 h, and
215 OD_{600} values of the wells were measured both before and after the incubation in a
216 Synergy H1 Hybrid plate reader (BioTec, Winooski, VT). The MIC of nisin was
217 determined as the lowest concentration that inhibited growth (defined as an OD_{600} value
218 ≥ 0.05). A total of three biological replicates were conducted.
219
220 **2.2 Pre-growth conditions and culture preparation**
221 **2.2.1 Selection of pre-growth conditions**
222 Based on survey results from several seafood processors and an on-site visit to a
223 processing facility of commercial smoked seafood, a series of pre-growth conditions were
224 selected to reflect the environments associated with either cold-smoked salmon finished
225 product or the processing facilities, including (i) 7°C BHI broth, (ii) 7°C BHI with pH=9
226 (1N NaOH), (iii) 7°C BHI with pH=6.1 (6N HCl), (iv) 7°C BHI with 4.65% water phase
227 (w.p.) NaCl, and (v) 7°C BHI with 0.5 μg/ml benzalkonium chloride.

228

229 **2.2.2 Growth curves and determination of growth phase**

230 In order to determine the time to reach mid-logarithmic (log) phase or early stationary
231 phase, growth curves were generated for each strain under each of the pre-growth
232 conditions. Sidearm flasks containing 100 ml of BHI broth adjusted to provide each of
233 the pre-growth conditions were cooled to 7°C prior to inoculation. Overnight cultures
234 were prepared for each individual strain and used to inoculate sidearm flasks at a 1:100
235 dilution. A 20D+ spectrophotometer (Thermo Fisher Scientific, Waltham, MA) with a
236 linear range from 0.2-0.7 was used to measure the optical density (OD_{600}) of the culture
237 in flasks immediately upon inoculation and every 24 h thereafter. When necessary, the
238 flask cultures were appropriately diluted to stay within the linear range of the
239 spectrophotometer.

240

241 **2.2.3 Culture preparation**

242 Prior to experiments in broth or on cold-smoked salmon, the frozen stock culture for each
243 of the strains was streaked onto BHI agar and incubated at 37°C for 20-24 h. A single
244 colony from a freshly streaked plate was inoculated into 5 ml BHI broth, followed by a
245 18 h incubation in shaking incubator (New Brunswick™ Innova® 43, Hamburg,
246 Germany) at 37°C and 230 rpm. For each strain, sterilized metal-cap flasks filled with
247 100 ml growth medium were prepared and pre-cooled to 7°C. The flasks were then
248 inoculated with 1 ml bacterial culture and subsequently incubated at 7°C until mid-log or
249 early-stationary phase was reached.

250

2.3 Evaluation of the efficacy of nisin treatment against *L. monocytogenes* in modified growth medium (SalBHI)

BHI broth was modified (SalBHI) to contain 4.65% w.p. NaCl and adjusted to pH = 6.1 to mimic the environment provided by commercially processed cold-smoked salmon (Stasiewicz et al., 2010). Filter sterilized SalBHI with or without 12.5 ppm nisin (0.25 g of Nisaplin per 500 ml of SalBHI) was aliquoted (5 ml) into sterile tubes. For each pre-growth condition, except 7°C BHI pH = 9, all six strains were pre-grown individually in metal-cap flasks as described above until mid-log or early-stationary phase. Once cultures reached the desired growth phase, SalBHI tubes both with and without nisin were inoculated at a final concentration of ~ 10^6 CFU/ml. Since none of the strains were able to grow in BHI pH=9 for the time period tested, in order to test a high pH stress, bacterial cells were grown at 7°C in regular BHI to mid-log or early-stationary phase and subsequently transferred into BHI adjusted to pH = 9 for a 30 min shock prior to SalBHI inoculation. Inoculated SalBHI tubes were incubated at 7°C for 24 h without shaking. The *L. monocytogenes* levels in the SalBHI tubes was then determined by serial dilution and spread plating on BHI agar plates followed by incubation at 37°C for 24 h. Colonies were enumerated using a SphereFlash® Automated Colony Counter (Neutec, Albuquerque, NM).

2.4 Evaluation of the efficacy of nisin treatment against *L. monocytogenes* on cold-smoked salmon

Cold-smoked salmon (Acme Smoked Fish Corporation), containing celery extract as the nitrite source, was pre-sliced and vacuum-packed in 600 g per package at their facility.

Packages of salmon from a single batch were obtained and stored at -20°C. Prior to each experiment, a single package was thawed overnight at 4°C. Based on the data from the SalBHI experiment, pre-growth condition/growth phase combinations which conferred the lowest nisin susceptibility and yielded the highest strain-to-strain variability were chosen. The pre-growth condition/growth phase combinations selected for experiments on cold-smoked salmon included (i) stationary phase in 7°C BHI 4.65% w.p. NaCl; (ii) stationary phase in 7°C BHI pH=6.1; (iii) log phase in 7°C BHI with 0.5 µg/ml benzalkonium chloride; and (iv) stationary phase in 7°C BHI as a control. For each strain, an overnight culture was sub-cultured (1:100) into a pre-cooled flask with 100 ml of the pre-growth medium. The inoculated flasks were incubated without shaking at 7°C until the cultures reached the targeted growth phase. For preparation of the salmon samples, inoculation of *L. monocytogenes*, and processing of samples, we followed the protocol previously described in Kang et al. (2014). For each experiment, 10 ± 0.5 g salmon slices were aseptically prepared for each of the six strains as well as a non-inoculated control, two treatments (untreated and nisin-treated) and three sampling days (1, 15, and 30) for a total of 42 samples. For nisin-treated salmon slices, 300 µl of a nisin solution (0.833 mg/ml) was applied evenly on the surface of the salmon slice for a final concentration of 0.025 mg/g or 25 ppm. To allow adsorption of nisin to the salmon surface, slices were incubated in a biosafety cabinet for 30 min (NuAire, Inc., Plymouth, MN). Bacterial cultures were inoculated onto the salmon slices at a target final concentration of approximately 10^6 CFU/g, followed by another 30 min incubation in the biosafety cabinet to allow attachment of the cells onto the salmon surface. All samples were then vacuum packed in whirl-pack filter bags (North America Sales Company, Inc., Pacific Palisades,

297 CA), incubated at 7°C, and processed after 1, 15 and 30 days of incubation. On each
298 processing day, salmon samples were diluted with 40 ml 1% peptone water and
299 homogenized by 1 min vigorous hand-massaging. Filtered salmon homogenate was
300 serially-diluted and spread-plated on MOX agar plates. Non-inoculated control samples
301 were plated on BHI plates in addition to MOX agar plates to monitor the level of native
302 microbiota in salmon. All MOX agar plates were incubated at 30°C for 48 h, and all BHI
303 plates were incubated at 37°C for 24 h. Colonies of *L. monocytogenes* as well as native
304 microbiota were enumerated using SphereFlash® Automated Colony Counter (Neutec,
305 Albuquerque, NM). Four biological replicates were conducted for each of the pre-growth
306 conditions.
307
308 **2.5 Statistical analysis**
309 All statistical analyses were performed in R Statistical Programming Environment (R
310 Core Team, 2018). *L. monocytogenes* count data (CFU/ml or CFU/g) were log
311 transformed for both the SalBHI experiments ($\log_{10}$ CFU/ml) and the salmon experiments
312 ($\log_{10}$ CFU/g).
313 For SalBHI experiments, log reduction was calculated as the log difference
314 between *L. monocytogenes* levels in untreated and nisin-treated samples. A linear mixed
315 effects model (SalBHI lme model) was constructed, using lme4 (v 1.1.17) package (Bates
316 et al., 2015), with log reduction as the response variable and pre-growth condition
317 (Condition), strain (Strain), and growth phase (Phase) as the fixed effects. Interactions
318 included in the model were the two-way interactions between (i) Condition and Phase, (ii)
319 Condition and Strain, and (iii) Strain and Phase, as well as the three-way interaction

among all fixed effects. Biological replicate was included in the model as a random effect. A three-way analysis of variance (ANOVA) was performed to the SalBHI lme model to explore the effects of pre-growth condition, strain, growth phase, as well as their interactions on the log reduction of *L. monocytogenes* due to nisin treatment in SalBHI. Upon the finding of any significant main effects or interactions, post hoc analysis was performed for further investigation using emmeans (v 1.4.4) package (Lenth, 2020).

For salmon experiments, log count data (log_{10} CFU/g) were directly fit with a linear mixed effects model (salmon lme model). The response of the model was the log_{10} CFU/g of *L. monocytogenes* on inoculated cold-smoked salmon samples. Fixed effects were: (i) nisin (Nisin), (ii) pre-growth condition (Condition), (iii) serotype (Serotype), (iv) source of the isolation (Source), and (v) storage day (Day). Two-way interactions included were all combinations of (i) nisin, (ii) condition, (iii) day, (iv) serotype, and (iv) source (e.g., interaction between nisin and condition). Three-way interactions included were those between (i) nisin, condition, and day, (ii) nisin, serotype, and source, and (iii) nisin, condition, and serotype. Age of salmon (duration of frozen storage at -20°C before thawing) was included in the model as a random effect. A three-way ANOVA was performed on the salmon lme model to explore the effect of pre-growth condition, serotype, source of isolation, storage day as well as their interactions on the nisin efficacy against *L. monocytogenes* on cold-smoked salmon. Post hoc analysis, including Tukey's HSD Test and Dunnett's correction, was conducted to investigate significant main effects or interactions based on the results of ANOVA.

The threshold of significance set for all statistical tests was $P = 0.05$. Raw data and R code used for data analysis of this study are available on GitHub (https://github.com/FSL-MQIP/PregrowthListeria_Nisin).

3. RESULTS

3.1 Characterization of seafood-relevant *L. monocytogenes* strains

A collection of six strains was assembled to represent the lineages (I and II) and serotypes (1/2a, 1/2b, and 4b) that are most commonly found in cold-smoked salmon RTE products and processing environments (Table 1). Strains were characterized by WGS, followed by identification of genes related to stress response, virulence, and tolerance to nisin and quaternary ammonium compound, and nisin MIC. A phylogenetic tree based on cgMLST confirmed serotype classification and showed that the six strains selected represented distinct clades (Supplemental Figure 1). Selected genes involved in general stress response, nisin resistance, and virulence were present in the genomes of all six strains with no premature stop codons (Table 1), except for *inlA* in both serotype 1/2a strains (FSL F2-0237 and FSL L4-0396). Although all of the strains harbor *mdrL*, a gene encoding an efflux pump that confers reduced sensitivity to benzalkonium chloride (Romanova et al., 2006), some strains also harbor additional quaternary ammonium compound tolerance genes such as *bcrABC* and *qacH* (Table 1). Lastly, all six strains used in this study showed comparable intrinsic nisin susceptibility with minimum inhibitory concentration (MIC) ranging from 0.0156 to 0.0313 mg/ml.

3.2 In SalBHI broth, pre-growth conditions affect nisin efficacy against *L. monocytogenes* in a strain- and growth phase- dependent manner

The effect of pre-growth condition on the nisin efficacy against *L. monocytogenes* grown to both log and stationary phase was initially investigated in BHI broth modified to represent the characteristics of cold-smoked salmon (SalBHI). *L. monocytogenes* numbers recovered from both untreated and nisin-treated cultures after 24 h were used to calculate the log reduction obtained due to nisin treatment. A linear mixed effects model (SalBHI lme model) was constructed to investigate the effect of pre-growth condition (Condition), growth phase (Phase), and strain (Strain) as well as their interactions on the log reduction of *L. monocytogenes* due to nisin treatment. The three-way interaction between Condition, Phase and Strain was found to be highly significant ($P < 0.001$), indicating that pre-growth condition affected nisin treatment in a strain- and growth phase- dependent manner (Table 2, Figure 1). The log reduction of *L. monocytogenes* numbers due to nisin treatment ranged from 0.37 (for strain FSL N1-0061 pre-grown to log phase in BHI with 0.5 μg/ml benzalkonium chloride) to 3.58 (for strain FSL L4-0060 pre-grown to log phase in BHI) (Figure 1). In general, pre-growth in the BHI control (to either log or stationary phase) provided the highest log reduction for most strains, with bacteria pre-grown to log and stationary phase showing similar log reductions (Figure 1). In contrast, the effect of pre-growth condition varied with growth phase for conditions other than the BHI control. For example, pre-growth to log phase in BHI 4.65% w.p. NaCl and in BHI pH = 6.1 resulted in higher log reductions for most strains as compared to pre-growth to stationary phase under the same condition.

385 To identify the pre-growth condition/growth phase combinations that rendered a
386 "worst case scenario" of lowest nisin reduction and highest strain-to-strain variability, the
387 mean and coefficient of variance of the log reduction were calculated for each pre-growth
388 condition/growth phase combination across strains (Table 3). Of all combinations tested,
389 pre-growth to stationary phase in BHI 4.65% w.p. NaCl, pre-growth to stationary phase
390 in BHI pH = 6.1, and pre-growth to log phase in BHI 0.5 µg/ml benzalkonium chloride
391 resulted in the lowest mean log reduction and the highest coefficient of variance across
392 strains (Table 3). These three combinations were therefore considered as the "worst case
393 scenarios" and selected for challenge studies on cold-smoked salmon. From here on,
394 these pre-growth condition/growth phase combinations will be simplistically referred to
395 as "NaCl", "pH", and "Quat", respectively. Since no significant difference ($P > 0.05$;
396 pairwise comparison) was identified between the two control pre-growth conditions (pre-
397 growth in BHI to log and stationary phase), pre-growth to stationary phase in BHI was
398 selected as the control condition for experiments on cold-smoked salmon and referred to
399 in the following sections as "BHI".
400
401 **3.3 Pre-growth conditions and strain diversity affect nisin efficacy against *L.***
402 ***monocytogenes* on cold-smoked salmon.**
403 After the initial experiments in SalBHI detailed above, the effect of pre-growth condition
404 and strain diversity on nisin efficacy against *L. monocytogenes* was then investigated on
405 cold-smoked salmon. While the numbers of *L. monocytogenes* recovered from salmon
406 were in general lower in nisin-treated samples compared to untreated samples, all strains
407 were able to grow throughout the 30-day incubation, even in the nisin-treated samples, to

408 levels comparable to, and sometimes indistinguishable from, the untreated samples
409 (Figure 2, Supplemental Table 1). A linear mixed effects model (salmon lme model) was
410 constructed to investigate the effect of pre-growth condition (Condition), day in storage
411 (Day), serotype (Serotype), source of isolation of the strains used (Source) as well as their
412 interactions on nisin efficacy against *L. monocytogenes* on cold-smoked salmon. The
413 model estimated log reduction due to nisin treatment, averaged across storage days 1, 15,
414 and 30, varied by pre-growth condition and strain, ranging from 0.42 for strain FSL F2-
415 0310 following pre-growth in "pH" to 1.79 for strain FSL L3-0051 following pre-growth
416 in "BHI" (Figure 2, Supplemental Table 2). Although most of the main effects and two-
417 way interactions were identified as significant (Table 4), further interpretation of them
418 was not deemed appropriate since they were all included in the three significant three-
419 way interactions, which will be discussed further in the following sections.
420
421 **3.3.1 The effect of pre-growth condition on nisin efficacy against *L. monocytogenes***
422 **on cold-smoked salmon varies with storage time**
423 The three-way interaction between Nisin, Condition, and Day was identified to be
424 significant ($P < 0.05$), indicating that pre-growth condition affects nisin efficacy against *L.*
425 *monocytogenes*, and that this effect varies throughout the storage (Table 4). For nisin-
426 treated samples, pre-growth in "NaCl", "pH", and "Quat" resulted in higher *L.*
427 *monocytogenes* levels on average across all strains on day 30 (~8.5 $\log_{10}$ CFU/g)
428 compared to pre-growth in "BHI" (~7.5 $\log_{10}$ CFU/g) (Supplemental Figure 2). In
429 contrast, *L. monocytogenes* levels in untreated samples were not affected by pre-growth
430 condition (Supplemental Figure 2). Log reduction between untreated and nisin-treated

samples across strains was then calculated from the model estimated log counts (log_{10} CFU/g) to represent the efficacy of the nisin treatment (Figure 3); a pairwise comparison analysis was performed to investigate the effect of pre-growth condition and storage day on nisin efficacy. While a significantly higher nisin efficacy was observed following pre-growth in "NaCl" at day 1 compared to "BHI" (adj. $P < 0.05$), nisin efficacy was significantly decreased by pre-growth in "pH" and "Quat" at day 15 and by all three pre-growth conditions at day 30 (Figure 3).

3.3.2 The effect of pre-growth condition on nisin efficacy against *L. monocytogenes* on cold-smoked salmon varies with serotype

The three-way interaction between Nisin, Serotype, and Source was also determined to be significant ($P < 0.05$), suggesting that the nisin efficacy against *L. monocytogenes* averaged by pre-growth condition and storage day is dependent on serotype and source of isolation. The *L. monocytogenes* levels in nisin-treated samples were similar across strains from the same serotype and across strains of different serotypes isolated from the same source type, except for the serotype 1/2b strain isolated from finished product (FSL L3-0051), which showed lower log counts when treated with nisin as compared to the other strains (Supplemental Figure 3). This observation was supported by a pairwise comparison of the model estimated log reduction (untreated versus nisin-treated samples) between *L. monocytogenes* strains with different serotypes, but the same source of isolation (Table 5), which showed that the serotype 1/2b strain isolated from finished product was significantly more susceptible to nisin compared to the 1/2a and 4b strains isolated from the same source type (adj. $P < 0.05$). However, the strains isolated from

454 environmental sources showed no difference in nisin susceptibility when different
455 serotypes were compared; for example, the 1/2a and the 1/2 b strain isolated from
456 environmental samples did not differ significantly in log reduction (see Table 5).
457 Although the difference in response observed for 1/2b strains in nisin-treated samples
458 suggests an effect of isolation source, it is important to note that, for each of the isolation
459 sources, only one strain from each serotype was used in this study; therefore the effect we
460 see for strain FSL L3-0051 might in fact be a strain effect and not a serotype effect.
461 The three-way interaction between Nisin, Condition and Serotype was also found
462 significant (Table 4), indicating that the effect of pre-growth condition on nisin efficacy
463 against *L. monocytogenes*, averaged across storage days and isolation sources, is
464 dependent on serotype, and in turn, the effect of serotype is also dependent on pre-growth
465 condition. Overall, the *L. monocytogenes* levels on nisin-treated samples varied
466 considerably with serotype and pre-growth condition (Supplemental Figure 4). Pairwise
467 comparison of the model estimated log reduction (untreated versus nisin-treated) across
468 pre-growth conditions revealed that different pre-growth conditions induced cross-
469 protection against nisin treatment for different serotypes of strains. For serotype 1/2a
470 strains, pre-growth in “pH” led to a significantly lower log reduction compared to “BHI”,
471 while for serotype 1/2b strains, a significantly lower log reduction was observed for pre-
472 growth in “Quat” (Figure 4). For serotype 4b strains, pre-growth in both “pH” and “Quat”
473 led to a significantly lower log reduction compared to “BHI” (Figure 4). In contrast, the
474 average log reduction across all serotypes was not affected by pre-growth in “NaCl”
475 (Figure 4).
476

4. DISCUSSION

L. monocytogenes contamination of RTE foods is a universal problem that can lead to health and economic repercussions, therefore, control of *L. monocytogenes* in RTE foods, including cold-smoked salmon, is of outmost importance. Our data show that pre-exposure to mildly acidic environment, high salt content, or a sublethal concentration of quaternary ammonium compounds is likely to provide cross-protection against subsequent nisin treatment of *L. monocytogenes* on cold-smoked salmon. In addition, strain serotype also affected nisin efficacy; following pre-exposure to mildly acidic environment, serotype 1/2b strains were more susceptible to nisin treatment on cold-smoked salmon, compared to serotype 1/2a and 4b strains. Overall, our results highlight the importance of including a good representation of strain diversity and considering environment-specific conditions for the development and validation of strategies for the control of *L. monocytogenes* on cold-smoked salmon.

4.1 Pre-exposure of L. monocytogenes to seafood-relevant conditions affects nisin treatment on cold-smoked salmon

Preliminary broth experiments indicated that pre-growth in mildly acidic pH ("pH"), high salt content ("NaCl"), and presence of a sub-lethal concentration of quaternary ammonium compounds ("Quat") yielded lowest nisin efficacy combined with highest strain-to-strain variability; these pre-growth conditions were selected for cold-smoked salmon experiments. *L. monocytogenes* showed reduced susceptibility to nisin treatment on cold-smoked salmon following pre-growth in "pH". This suggests that pre-exposure of *L. monocytogenes* to mildly acidic environment, either in the product or in the processing

500 facility, might render nisin treatment less efficient in killing this pathogen. This finding is
501 consistent with previous studies in liquid broth (Kang et al., 2015; van Schaik et al.,
502 1999). For example, Kang et al (2015) showed that pre-exposure to 2% potassium lactate
503 (pH=6.0) rendered *L. monocytogenes* more resistant to nisin (Kang et al., 2015). In a
504 separate study, acid adapted (pre-grown in liquid medium adjusted to pH=5.5 using lactic
505 acid) *L. monocytogenes* cells also showed elevated resistance to nisin (van Schaik et al.,
506 1999). Acid adaptation of *L. monocytogenes* has been shown to involve alterations of cell
507 wall and cell membrane resulting in a decrease in membrane fluidity (Mastronicolis et al.,
508 2010), which has been shown to decrease nisin susceptibility (Crandall and Montville,
509 1998; Davies et al., 1996; Mazzotta and Montville, 1997; Ming and Daeschel, 1993,
510 1995). Our data as well as others thus suggest that there is a potential cross-protection
511 between acid stress and nisin resistance, which might impact the effective use of this
512 bacteriocin in mildly acidic food products such as cold-smoked salmon. Therefore, the
513 incorporation of acid adapted *L. monocytogenes* cells in challenge studies would provide
514 good assessment of worst-case scenarios.
515 The effect of pre-growth in "NaCl" on *L. monocytogenes* numbers on nisin-
516 treated samples varied throughout shelf life. At day 1, the difference in numbers between
517 nisin-treated and untreated cells was significantly larger after pre-growth in "NaCl",
518 compared to "BHI", indicating a possible enhanced killing of *L. monocytogenes* pre-
519 exposed to salt stress. However, at day 30, the difference in *L. monocytogenes* levels
520 between untreated and nisin-treated samples was significantly smaller following pre-
521 growth in "NaCl" compared to "BHI". A number of scenarios could explain this
522 observation, for example, pre-growth in salt could have led to a large number of injured

523 cells that were not detectable on day 1 but were then able to fully recover and grow
524 within the 30-day storage. Cross-protection against nisin after osmotic stress has been
525 previously reported (Bergholz et al., 2013; De Martinis et al., 1997). For example,
526 Bergholz et al (2013) showed in liquid medium that *L. monocytogenes* cells exposed to
527 osmotic stress (6% NaCl) exhibited increased capability to survive a subsequent nisin
528 challenge at 7°C, and that this osmotic stress-induced nisin resistance was at least
529 partially mediated by σ^B and LiaR (Bergholz et al., 2013). As salt is typically used for the
530 preservation of RTE food products, such as cold-smoked salmon, the fact that osmotic
531 stress might provide cross-protection to nisin may negatively affect the efficacy of this
532 antimicrobial in the seafood industry, indicating that assessment of nisin efficiency in the
533 seafood industry should include pre-exposure to salt.
534 Cross-protection to nisin was also observed following pre-growth under presence
535 of sublethal concentrations of benzalkonium chloride, a type of quaternary ammonium
536 compound frequently used in sanitizers in food industries (Sidhu et al., 2002). Other
537 studies have also suggested that use of benzalkonium chloride in food processing
538 facilities may provide for cross-protection of *L. monocytogenes* against other stresses
539 (Lundén et al., 2003; Ratani et al., 2012; Romanova et al., 2006). Several mechanisms
540 have been proposed to provide resistance to benzalkonium chloride including (i) changes
541 of cell surface permeability that increase resistance non-specifically (Mereghetti et al.,
542 2000; Romanova et al., 2006; To et al., 2002), and more frequently, (ii) the acquisition of
543 efflux pumps such as those encoded by *mdrL*, *bcrABC*, and *qacH* (Elhanafi et al., 2010;
544 Mata et al., 2000; Müller et al., 2013). While the strains used in this study varied in the
545 number of benzalkonium chloride resistance mechanisms they possessed, they all

546 exhibited similarly reduced nisin susceptibility following pre-growth in “Quat”. Previous
547 work has shown that upon adaptation to benzalkonium chloride, the cell membrane of
548 benzalkonium chloride resistant strains is modified to possess an increased proportion of
549 longer fatty acids and thus less fluidity (To et al., 2002), a modification that might
550 contribute to the cross-protection to nisin. Altogether, the cross-protection to nisin
551 induced by pre-growth of *L. monocytogenes* in “Quat” is likely to involve multiple
552 adaptation mechanisms as well as some unidentified regulatory pathways. Therefore, the
553 use of *L. monocytogenes* cells adapted to sublethal concentrations of this commonly used
554 sanitizer should be considered in challenge and validation studies of strategies to control
555 this pathogen.
556
557 ***4.2 L. monocytogenes serotype affects nisin treatment on cold-smoked salmon***
558 While the preliminary results obtained in SalBHI experiments seemed to suggest that *L.*
559 *monocytogenes* strains showed different levels of nisin susceptibility both within and
560 between pre-growth conditions, in cold-smoked salmon experiments, the cross-protection
561 induced by pre-growth in “NaCl” and “Quat” seemed to be universally applicable to all
562 strains. However, the cross-protection induced by “pH” was significantly affected by
563 strain, more specifically by serotype. This observed effect is consistent with the results
564 from SalBHI experiments, which showed that, for all pre-growth and growth phase
565 combinations, the most susceptible strain was either FSL L3-0051 or FSL L4-0060, both
566 of which belong to serotype 1/2b. It has long been recognized that *L. monocytogenes*
567 strains differ considerably in their responses to different types of stress (Barmpalia-Davis
568 et al., 2008; Bergholz et al., 2010; Tang et al., 2013). For example, lineage-dependent

569 sensitivity to different stresses, including gastric environment (Barmpalia-Davis et al.,
570 2008), salt (Bergholz et al., 2010), and nisin (Tang et al., 2013), has previously been
571 reported for *L. monocytogenes*. Moreover, a study comparing the response to meat and
572 sausage juices of three *L. monocytogenes* strains showed across strains differences in the
573 expression levels of *gadC* and *gadE*, genes responsible for acid adaptation (Rantsiou et
574 al., 2012). This suggests that different strains may incorporate different regulatory
575 mechanisms to address the same environmental stresses, which in turn makes them vary
576 in ability to cope with any subsequent stresses. Although no study has evaluated the
577 strain-to-strain variability in nisin susceptibility due to pre-growth in "pH" on cold-
578 smoked salmon, our findings suggest that *L. monocytogenes* strains might react
579 differently to pre-exposure to mildly acidic environments, and therefore may differ in
580 acid-induced cross-protection against nisin.

581 Overall, our data highlight the importance of considering the effect of relevant
582 environmental conditions *L. monocytogenes* are exposed to in both the processing
583 environment (e.g., sanitizers) as well as those intrinsic to the product (e.g., high salt,
584 mildly acidic pH) when assessing the efficacy of control strategies (e.g., nisin treatment)
585 for *L. monocytogenes*. In addition, *L. monocytogenes* strains vary in their response to
586 various stresses, which may lead to different levels of cross-protection that diminish the
587 efficacy of a control strategy. Taken together, the incorporation of worst-case scenarios,
588 such as those suggested in this study, as well as a broad range of strains encompassing
589 those most commonly associated with RTE seafood, would be ideal for studies on
590 development and validation of *L. monocytogenes* control strategies. Furthermore,
591 information generated from studies like this one may also be incorporated to improve

predictive modeling tools for the estimation of appropriate antimicrobial concentrations or product reformulations.

5. CONFLICT OF INTEREST

Declarations of interest: none.

6. CREDIT AUTHOR STATEMENT

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7. ACKNOWLEDGEMENTS

This publication is a product resulting from project R/SHH-18 funded under award NA18OAR4170096 from the National Sea Grant College Program of the U.S. Department of Commerce's National Oceanic and Atmospheric Administration, to the Research Foundation for State University of New York Sea Grant. The statements, findings, conclusions, views and recommendations are those of the author(s) and do not necessarily reflect the views of any of those organizations. We thank Sarah Murphy

615 (Cornell Food Safety Laboratory) and Dr. Erika Mudrak (from the Cornell Statistical
616 Consultant Unit) for advice about statistical analysis. We are also grateful for the
617 technical assistance from Maureen Gunderson, Alan Bitar, and Liping Li.
618

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7. TABLES AND FIGURE LEGENDS

Table 1. *L. monocytogenes* strains used in this study

FSL number[a]	SRA[b]	Serotype	Ribotype	Genes with Premature Stop Codons[c]	Quat tolerance genes[d]	Nisin MIC[e] (mg/ml)	Source of isolation	Year	Reference
Lineage I									
FSL L3-0051	SRR6795895	1/2b	DUP-1042C	None	*mdrL*	0.0156	finished RTE food product (salmon)	2002	Sauders et al., 2004
FSL L4-0060	SRR6796400	1/2b	DUP-1043A	None	*bcrABC, mdrL*	0.0156	environment (drain) seafood plant	2002	Lappi et al., 2004
FSL F2-0310	SRR6796397	4b	DUP-1038B	None	*qacH, mdrL*	0.0156	finished RTE food product (salmon)	2000	Sauders et al., 2004
FSL N1-0061	SRR6796398	4b	DUP-1044A	None	*mdrL*	0.0156	environment (brine) seafood plant	1998	Norton et al., 2001
Lineage II									
FSL F2-0237	SRR6796395	1/2a	DUP-1062D	*inlA*	*qacH, mdrL*	0.0313	finished RTE food product (salmon)	1999	Sauders et al., 2004
FSL L4-0396	SRR6795875	1/2a	DUP-1039C	*inlA*	*bcrABC, mdrL*	0.0313	environment (drain) seafood plant	2002	Lappi et al., 2004

[a]Food Safety Lab (FSL) isolate information can be found on Food Micro Tracker, available at: http://www.foodmicrobetracker.com/.

[b]Sequence read archive (SRA) accession number.

[c]The sequence of the genes *inlA, prfA, sigB, rsbV, virR, virS, dltA*, and *mprF* was examined for the presence of premature stop codons using BLASTX.

[d]The genomes of all strains were explored using BLASTN for the presence of Quat (benzalkonium chloride) tolerance genes (*bcrABC, qacH, qacA, emrC, emrE,* and *mdrL*).

[e]Minimum inhibitory concentration of nisin.

Table 2. Three-way ANOVA summary of the SalBHI lme model

	Sum Sq[a]	Mean Sq[a]	NumDF[a]	DenDF[a]	F value[a]	Pr(>F)[a]
Condition	5.061	1.265	4	30.484	7.906	<0.001
Phase	1.084	1.084	1	30.472	6.773	0.014
Strain	31.352	6.271	5	150.057	39.184	<0.001
Condition:Phase	6.539	1.635	4	30.484	10.216	<0.001
Condition:Strain	18.037	0.902	20	149.996	5.636	<0.001
Phase:Strain	0.838	0.168	5	150.057	1.047	0.392
Condition:Phase:Strain	9.777	0.489	20	149.996	3.055	<0.001

[a]Three-way ANOVA statistics of the SalBHI lme model by reference coding (R default). Sum sq: the sum of squares due to the factor or interaction; Mean sq: mean of the sum of squares due to the factor or interaction; NumDF: numerator degree of freedom; DenDF: denominator degree of freedom; F value: the F-statistic; Pr(>F): the *P*-value.

859 Table 3. Effect of pre-growth condition on nisin efficacy (log reduction) against

860 *L. monocytogenes* in SalBHI

Pre-growth Condition	Growth Phase	Mean log reduction[a]	Coefficient of Variance[b]	Pairwise Comparison *P*-value[c]
7°C BHI[d]	Log	2.81	0.175	NA
7°C BHI 4.65% water phase (w.p.) NaCl	Log	2.44	0.218	0.492
7°C BHI pH = 6.1	Log	2.63	0.214	0.889
7°C BHI 0.5 μg/ml benzalkonium chloride	Log	1.87	0.499	0.007
7°C BHI pH = 9[e]	Log	2.20	0.313	0.117
7°C BHI[d]	Stationary	2.66	0.128	NA
7°C BHI 4.65% water phase (w.p.) NaCl	Stationary	1.71	0.526	0.003
7°C BHI pH = 6.1	Stationary	1.34	0.559	<0.001
7°C BHI 0.5 μg/ml benzalkonium chloride	Stationary	2.24	0.192	0.356
7°C BHI pH = 9[a]	Stationary	2.65	0.176	1.000

861 [a]Estimated mean of log reduction across strains calculated for each pre-growth condition and growth phase combination
862 using emmeans (v 1.4.4) package (Lenth, 2020).
863 [b]Coefficient of variance of the estimated log reduction across strains calculated for each of the pre-growth condition and
864 growth phase combinations using goeveg (v 0.4.2) package (Goral and Schellenberg, 2018).
865 [c]*P*-value of pairwise comparison of log reductions among pre-growth conditions, within the same growth phase,
866 adjusted using Dunnett's method specifying 7°C BHI as the reference level; NA: not applicable.
867 [d]Pairwise comparison of log reduction was performed between the two control pre-growth conditions (log vs stationary)
868 without *P*-value adjustment. No significance was found between the conditions ($P > 0.05$).
869 [e]For 7°C BHI pH = 9, bacterial cells were grown in 7°C BHI and subsequently shocked in BHI adjusted to pH = 9 for
870 30 min.

871 Table 4. Three-way ANOVA summary of the salmon lme model

	Sum Sq[a]	Mean Sq[a]	NumDF[a]	DenDF[a]	F value[a]	Pr(>F)[a]
Nisin	185.13	185.13	1	560	691.87	<0.001
Condition	1.64	0.55	3	16	2.05	0.148
Serotype	2.89	1.44	2	560	5.39	0.005
Day	1182.44	541.22	2	560	2022.66	<0.001
Source	2.15	2.15	1	560	8.02	0.005
Nisin:Condition	11.30	3.77	3	560	14.08	<0.001
Nisin:Serotype	4.06	2.03	2	560	7.60	<0.001
Condition:Serotype	4.88	0.81	6	560	3.04	0.006
Nisin:Day	7.04	3.52	2	560	13.16	<0.001
Condition:Day	9.34	1.56	6	560	5.82	<0.001
Nisin:Source	0.23	0.23	1	560	0.88	0.349
Serotype:Source	6.22	3.11	2	560	11.61	<0.001
Nisin:Condition:Serotype	3.92	0.65	6	560	2.44	0.024
Nisin:Condition:Day	6.28	1.05	6	560	3.91	<0.001
Nisin:Serotype:Source	2.16	1.08	2	560	4.04	0.018

872 [a]Three-way ANOVA statistics of the salmon lme model by reference coding (R default). Sum sq: the sum of squares due to the

873 factor or interaction; Mean sq: mean of the sum of squares due to the factor or interaction; NumDF: numerator degree of freedom;

874 DenDF: denominator degree of freedom; F value: the F-statistic; Pr(>F): the *P*-value.

875

Table 5. Effect of serotype and source of isolation on nisin efficacy against *L. monocytogenes* on cold-smoked salmon

Isolation Source	Log Reduction[a]			1/2a - 1/2b		1/2a - 4b		1/2b - 4b	
	1/2a	1/2b	4b	Difference[b]	*P*-value[c]	Difference[b]	*P*-value[c]	Difference[b]	*P*-value[c]
Environment	1.08	1.16	1.04	-0.08	0.876	0.05	0.950	0.12	0.707
Finished Product	0.95	1.58	0.99	-0.64	<0.001	-0.05	0.948	0.59	<0.001

[a]Log reduction (untreated vs nisin-treated) estimated from the salmon lme model for each serotype and source of isolation, averaged across pre-growth conditions and storage days.

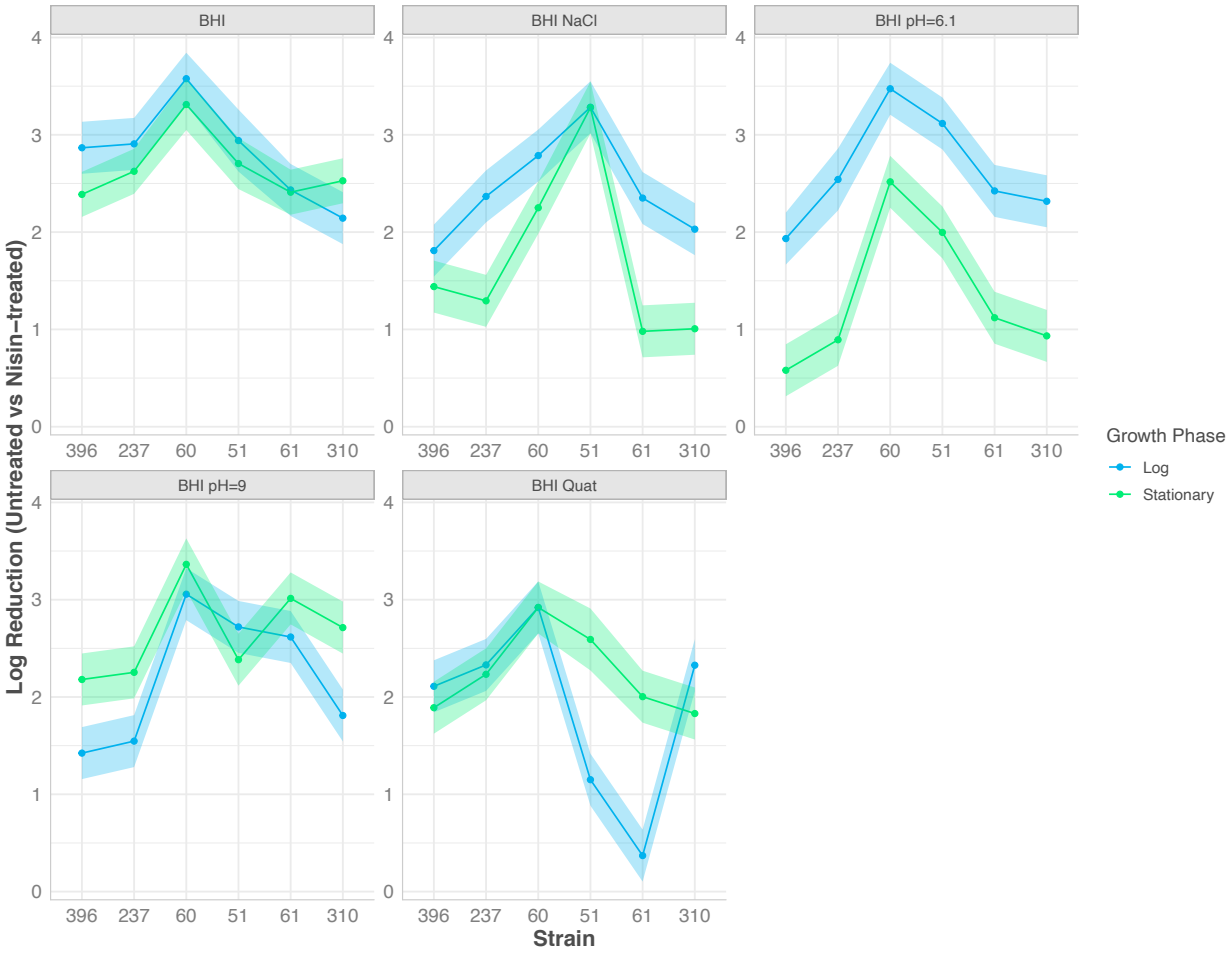
[b]Difference in log reduction between strains from different serotypes isolated from the same source type.

[c]*P*-value of the pairwise comparison between strains from different serotypes isolated from the same source type (overall $\alpha = 0.05$, Tukey's HSD).

883 **Figure 1.** Effect of pre-growth condition on nisin susceptibility of *L. monocytogenes*
884 strains in SalBHI after pre-growth to either log phase (blue) or stationary phase (green).
885 Prior to subculture into SalBHI, cells were pre-grown in 7°C BHI broth (BHI), 7°C BHI
886 4.65% w.p. NaCl (BHI NaCl), 7°C BHI pH = 6.1 (BHI pH=6.1), and 7°C BHI 0.5 µg/ml
887 benzalkonium chloride (BHI Quat), or pre-grown in 7°C BHI broth followed by 30 min
888 shock in BHI pH = 9 (BHI pH=9). For each of the strains, pre-growth conditions and
889 growth phases, means (solid circles) and standard errors (n=3, shaded area) were
890 estimated from the SalBHI lme model using emmeans (v 1.4.4) package (Lenth, 2020).
891 Due to space limitation, strain identification numbers were abbreviated as follows: 396=
892 FSL L4-0396, 237= FSL F2-0237, 60= FSL L4-0060, 51= FSL L3-0051, 61= FSL N1-
893 0061, and 310= FSL F2-0310.
894
895 **Figure 2.** *L. monocytogenes* levels (log_{10} CFU/g) on cold-smoked salmon stored at 7°C
896 for 30 days. Prior to salmon inoculation, cells were pre-grown in BHI broth (BHI), BHI
897 4.65% w.p. NaCl (NaCl), BHI pH = 6.1 (pH), and BHI 0.5 µg/ml benzalkonium chloride
898 (Quat). For each strain, pre-growth condition and day tested, raw data points were plotted
899 as standalone diamonds (n = 4) and estimated means from the salmon lme model were
900 plotted as solid circles connected by solid lines. Diamonds, circles and lines in blue and
901 green represent the bacterial level in untreated and nisin-treated samples, respectively.
902 Within the same panel (i.e. a single pre-growth condition and strain combination),
903 estimated means sharing one or more identical letters are not significantly different from
904 each other (overall $\alpha = 0.05$, Tukey's HSD).
905

Figure 3. Effect of pre-growth condition and storage day on nisin efficacy against *L. monocytogenes* on cold-smoked salmon. Pre-growth conditions included: BHI broth (BHI), BHI 4.65% w.p. NaCl (NaCl), BHI pH = 6.1 (pH), and BHI 0.5μg/ml benzalkonium chloride (Quat). For each pre-growth condition and storage day, raw data were summarized as means (diamond points) ± standard errors (error bars) across strains, and salmon lme model estimates were averaged across strains and plotted as color-coded bars. Within the same panel, pairwise comparison of the model estimates between pre-growth conditions was performed using Dunnett's method specifying "BHI" as the reference level. Significant codes: "***" adjusted $P < 0.001$; "**" $0.001 \leq$ adjusted $P < 0.01$; "*" $0.01 \leq$ adjusted $P < 0.05$; all comparisons that were significantly different are indicated.

Figure 4. Effect of pre-growth condition and serotype on nisin efficacy against *L. monocytogenes* on cold-smoked salmon. Pre-growth conditions included: BHI broth (BHI), BHI 4.65% w.p. NaCl (NaCl), BHI pH = 6.1 (pH), and BHI 0.5μg/ml benzalkonium chloride (Quat). For each serotype and pre-growth condition, raw data were summarized as means (diamond points) ± standard errors (error bars) across storage days and sources of isolation, and model estimates were averaged across storage days and sources of isolation and plotted as color-coded bars. Within the same panel, pairwise comparison of the model estimates between pre-growth conditions was performed using Dunnett's method specifying "BHI" as the reference level. Significant codes: "***" adjusted $P < 0.001$; "**" $0.001 \leq$ adjusted $P < 0.01$; "*" $0.01 \leq$ adjusted $P < 0.05$; all comparisons that were significantly different are indicated.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BHI NaCl
BHI pH=6.1
BHI pH=9
BHI Quat
Log Reduction (Untreated vs Nisin−treated)
Strain
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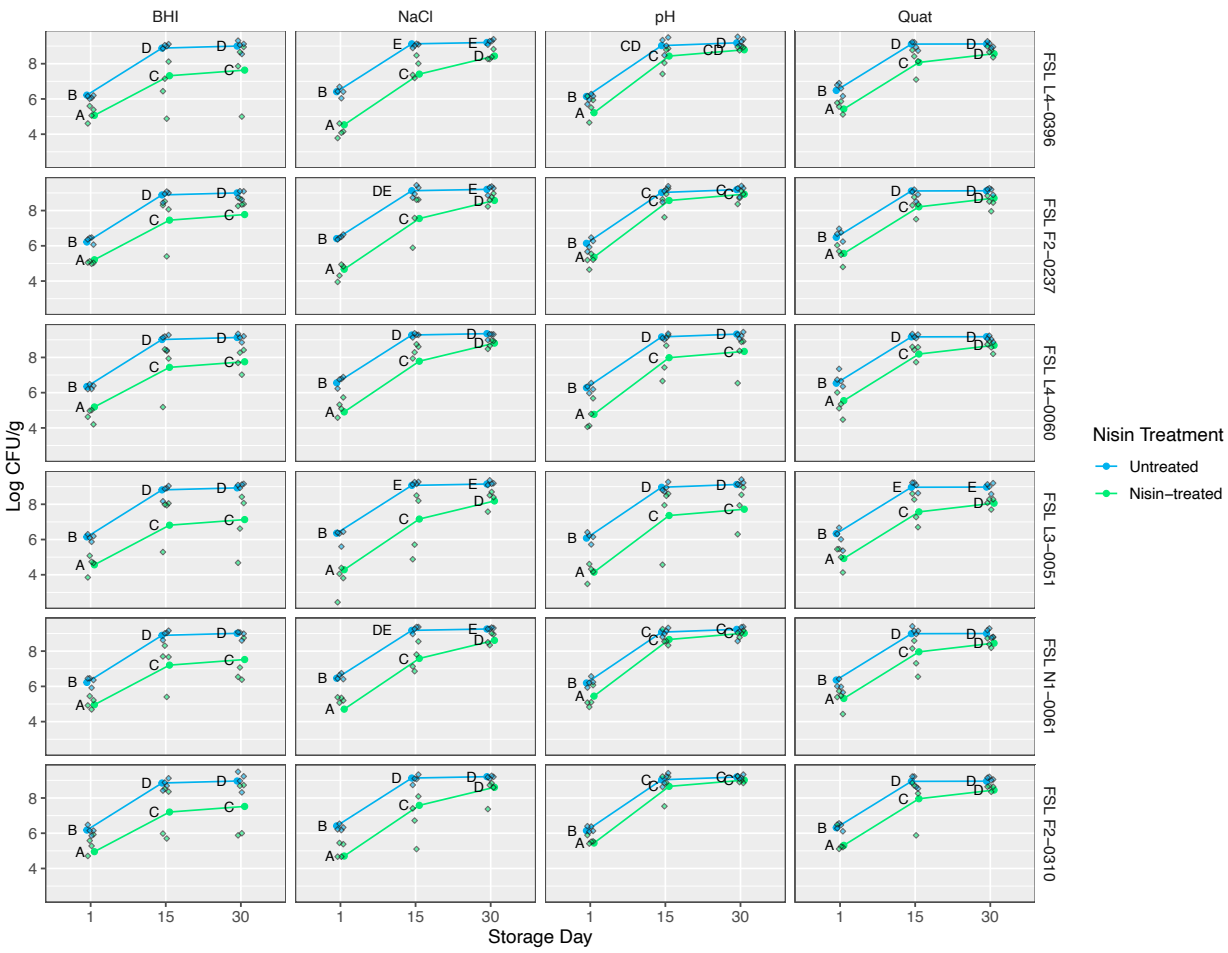
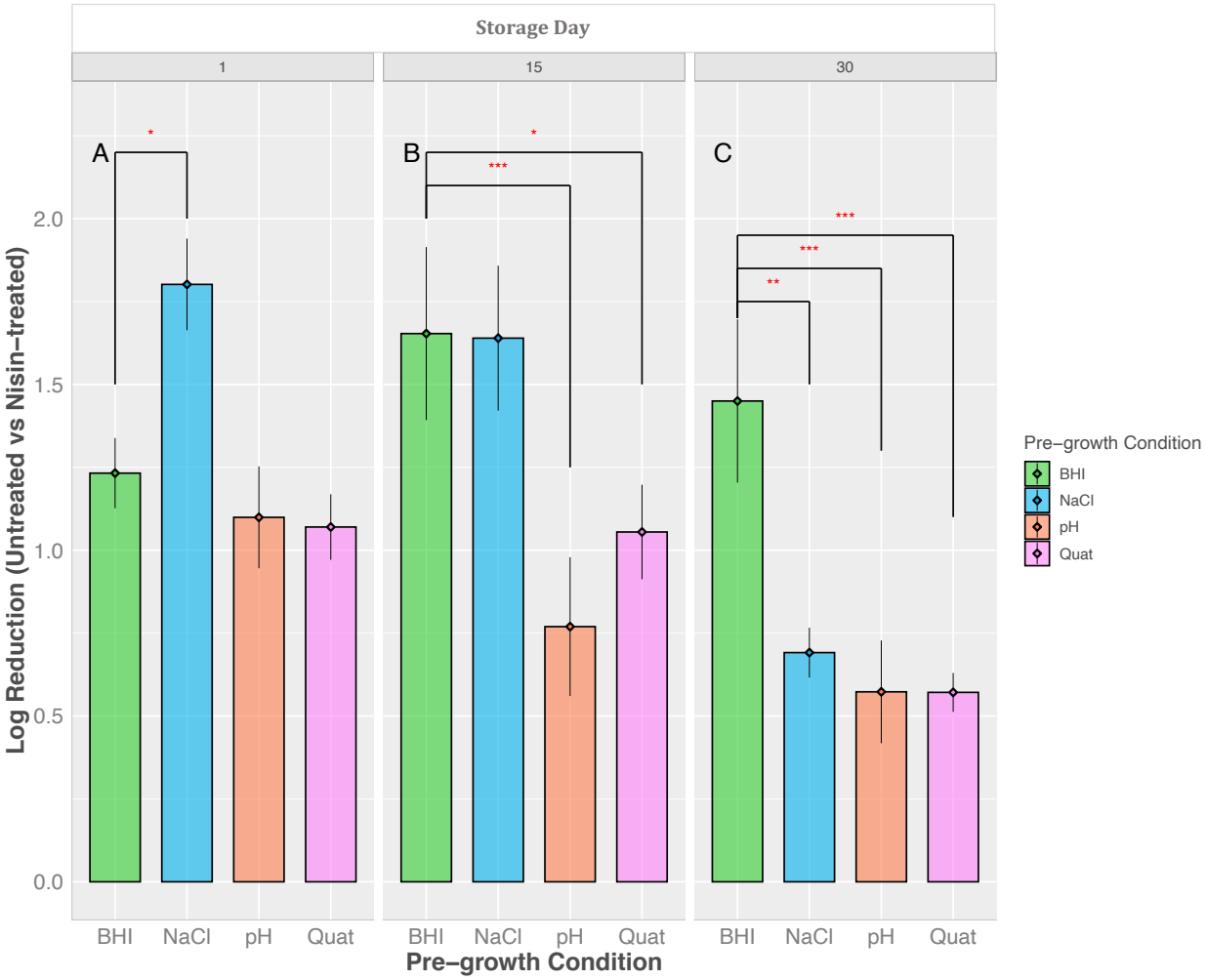
237
60
51
61
310
0
1
2
3
4
Growth Phase
Log
Stationary


BHI
NaCl
pH
Quat
FSL L4−0396
FSL F2−0237
FSL L4−0060
FSL L3−0051
FSL N1−0061
FSL F2−0310
Log CFU/g
Storage Day
Nisin Treatment
Untreated
Nisin−treated


Storage Day
1
15
30
A
B
C
*
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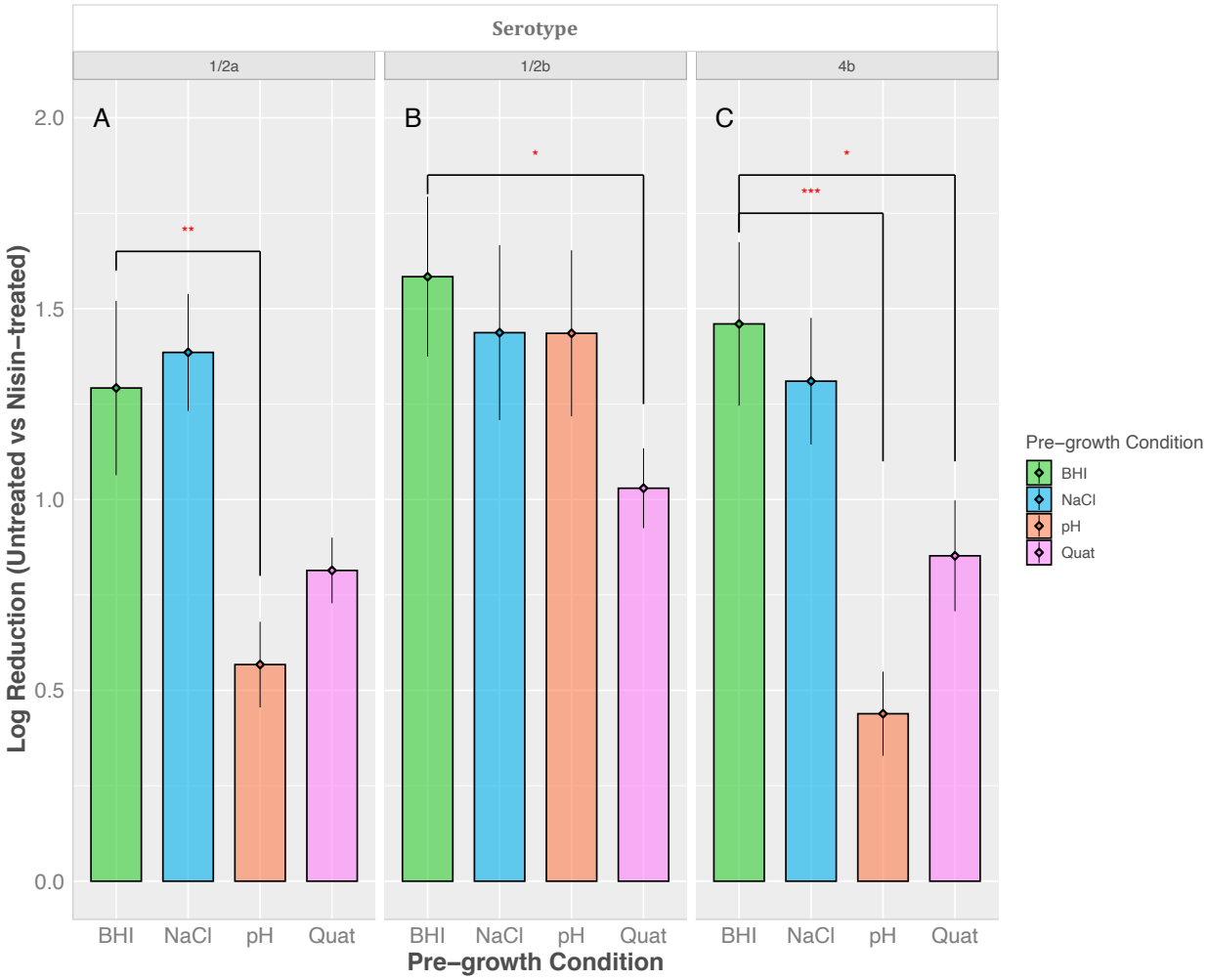
2.0
1.5
1.0
0.5
0.0
Log Reduction (Untreated vs Nisin−treated)
BHI
NaCl
pH
Quat
Pre−growth Condition
Pre−growth Condition
BHI
NaCl
pH
Quat


Serotype
1/2a
1/2b
4b
A
B
C
2.0
1.5
1.0
0.5
0.0
**
*
*
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Log Reduction (Untreated vs Nisin−treated)
BHI
NaCl
pH
Quat
Pre−growth Condition
Pre−growth Condition
BHI
NaCl
pH
Quat