SYNERGISTIC REDUCTION OF *LISTERIA MONOCYTOGENES* COLD GROWTH BY LACTATE AND DIACETATE

A Thesis

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by

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ABSTRACT

Listeria monocytogenes is a psychrotolerant foodborne pathogen that is of particular concern to the ready-to-eat meat and seafood industries because it's ability to grow at low temperatures reduces the ability of refrigeration to control the pathogen's growth in foods. Because *L. monocytogenes* generally contaminates foods at low levels and has a high infectious dose, the ability for the organism to grow in foods is critical for its ability to cause disease. One method to control *L. monocytogenes'* growth, widely used in the ready-to-eat meat industry, is to formulate products with combinations of the organic acids lactate and diacetate, as they appear to have synergistic (greater than additive) effects. The purpose of this work was to demonstrate synergistic growth inhibition by lactate and diacetate in a statistically rigorous manner. The approach was to grow strains of *L. monocytogenes* representing the two major genetic lineages in broth treated with lactate, diacetate or the combination of both at the same level at refrigeration temperature. The data show the growth inhibitor combination extends lag phase and decreases maximum growth rate similarly across both genetic lineages, and in a statistically significant manner (a significant interaction between treatments), quantitatively proving synergism. Monte Carlo simulations provided further evidence for synergy by predicting significantly slower growth to nominal endpoints, such as a 1-log increase, for the combination of inhibitors. This study quantitatively confirms the qualitative evidence in the literature for lactate and diacetate synergy and justifies the combined use in food product formulations. The final chapter describes two directions to continue this research, specifically (i) using these synergy calculations methods to characterize and optimize novel growth inhibiting treatment combinations and (ii) using molecular genetics techniques to investigate the mechanisms behind the synergistic actions of lactate and diacetate.

BIOGRAPHICAL SKETCH

Matthew Stasiewicz was born to Duane and Nancy Stasiewicz on May 8th, 1985 in Detroit, Michigan, then the family moved to Grosse Pointe Woods, Michigan where he attended Grosse Ponte North High School. Matt went to Michigan State University for undergraduate study earning both a B.S. in Biosystems Engineering, focusing on food process engineering, and a B.A. in Philosophy, focusing on ethics. During undergraduate studies he was mentored by Dr. Brad Marks on a research project to model the adaption of *Salmonella* to sublethal thermal heating as it affects subsequent thermal death rates, and therefore process safety. After undergraduate studies Matt interned with Kellogg Company where Louis Morel supervised work on pilot scale cereal processing refinement. Matt then came to Cornell for Master's studies with Dr. Martin Wiedmann to understand microbial factors behind food safety, leading to this body of work. After this project, Matt will continue working with Dr. Wiedmann for a PhD but with focused shifted from microbiology problems to risk assessment and application of mathematical models to food safety problems.

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CHAPTER 1

THE COMBINATION OF LACTATE AND DIACETATE SYNERGISTICALLY REDUCES COLD GROWTH IN BRAIN HEART INFUSION BROTH ACROSS LISTERIA MONOCYTOGENES LINEAGES

Abstract

Combinations of organic acids are often used in ready-to-eat foods to control the growth of *Listeria monocytogenes* during refrigerated storage. The purpose of this study was to quantitatively assess synergy between two organic acid growth inhibitors under conditions similar to those present in cold smoked salmon, and to assess the effect of evolutionary lineage on response to those growth inhibitors. Thirteen strains of *L. monocytogenes*, representing lineages I and II, were grown at 7°C in broth at pH 6.1 and 4.65% water-phase NaCl which was supplemented with 2% potassium lactate, 0.14% sodium diacetate, or the combination of both at the same levels. Our data suggest that lineages adapt similarly to these inhibitors, as the only significant growth parameter difference between lineages was a minor effect (±0.16 day, $p=0.0499$) on lag phase (λ). For all strains, lactate significantly extended λ, from 2.6±0.4 to 3.8±0.5 days ($p<0.001$), and lowered maximum growth rate (μ_{max}), from 0.54±0.06 to 0.49±0.04 $\log_{10}$(CFU/mL)/day ($p<0.001$), compared to the control. Diacetate was ineffective alone, but in combination with lactate, synergistically increased λ to 6.6±1.6 days ($p<0.001$) and decreased μ_{max} to 0.34±0.05 $\log_{10}$(CFU/mL)/day ($p<0.001$). Monte Carlo simulations provided further evidence for synergy between diacetate and lactate by predicting signficantly slower growth to nominal endpoints for the combination of inhibitors. This study shows potassium lactate and sodium diacetate have significant synergistic effects on both λ and μ_{max} of *L. monocytogenes* at refrigeration temperature in broth and justifies combining these inhibitors, at effective levels, in food product formulations.

Introduction

Listeria monocytogenes is an opportunistic pathogen that causes human illness almost exclusively from foodborne transmission *(24, 47)*. It possesses heat, acid, and osmotic stress response systems, which help it to survive on foods during processing as well as during transmission through a human or animal host *(41)*. Additionally, it is psychrotolerant, having the ability to grow at temperatures as low as -0.4°C *(51)*, therefore reducing the ability of refrigeration to inhibit *L. monocytogenes* growth. The species *L. monocytogenes* can be divided into at least three genetic lineages *(53)* with known differences in virulence *(7)*, gene expression *(38)*, growth, and survival during thermal inactivation *(10)*. Further, genetic lineages are differentially associated with human (lineage I), or environmental and food (lineage II) prevalence *(15)*.

L. monocytogenes contamination in ready to eat (RTE) foods is usually at low levels *(14)* and a large infectious dose is typically required to cause human disease *(46)*. Therefore, the ability of *L. monocytogenes* to multiply in foods is critical for its ability to cause disease *(8)*. The organism cannot grow in foods that: are stored frozen, have a pH $\leq$ 4.4, have an a_w < 0.92 or that have an effective growth inhibiting measure incorporated during production *(44)*. In situations where growth cannot be prevented altogether, strategies that reduce or prevent the ability of *L. monocytogenes* to grow in foods are predicted to significantly reduce listeriosis due to consumption of those foods *(45)*.

Consumption of RTE smoked seafood products has been identified as having a high risk per serving for listeriosis *(45)*. Cold smoked seafood products typically contain between 2.5 and 6% water phase salt, have a pH > 5.0, and contain sub-inhibitory levels of nitrites (100-200 ppm) and phenol compounds *(17)*. Smoked seafood products also have a high prevalence of *L. monocytogenes (14)*, most frequently through post-processing contamination at the processing plant, though *L.*

monocytogenes present in raw product can survive the cold smoking process and contribute to contamination *(17)*. The combination of growth-permissive product formulations and high rates of contamination create high risk per serving for listeriosis because when cold smoked seafood products are contaminated, *L. monocytogenes* can then grow to dangerous levels during refrigerated storage of the product *(45, 54)*.

In the RTE meat industry, natural and synthetic growth inhibitors (GI) have been used to control *L. monocytogenes* growth, and these methods are beginning to be investigated for use in cold smoked seafood products *(20)*. Often, GI are used in pairs, as research has shown that some combinations are more effective together than either of the inhibitors used alone *(12, 13)*. Yet, no standard method exists to evaluate the effects of single growth inhibitors or determine synergies when they are used in combination. Previously used methods include: measuring growth of *L. monocytogenes* over time in model systems *(37)*, high throughput screens for single or combination treatments that inhibit short-term growth *(16, 27)*, and challenge studies measuring growth in actual food products *(31, 50, 54)*. In studies where synergies between GI have been quantitatively demonstrated, they are commonly evaluated by showing the effects of a combination of GI are great than the sum of the individual effects *(31, 37)*, or by showing that the inhibitors have statistical interaction effects on the indicator for growth inhibition *(1, 30)*.

Combinations of the organic acid GI potassium lactate and sodium diacetate are widely used in the RTE meat industry *(42)*, which has resulted in commercial production of at least one lactate and diacetate blend (PURASAL OptiForm PD-Plus, PURAC America, Inc) that has also been evaluated for use in the cold smoked seafood industry *(31, 50)*. Yet, to the best of our knowledge, the synergistic action of potassium lactate and sodium diacetate on *L. monocytogenes* growth has never been demonstrated quantitatively. The purpose of this study was to evaluate potential

synergistic effects of potassium lactate and sodium diacetate on inhibition of *L. monocytogenes* grown at 7°C in broth media adjusted to the water phase NaCl and pH of cold smoked salmon, with the aim of demonstrating synergy quantitatively. Inhibitors were tested using strains from both lineage I and lineage II to determine if there were growth differences between these lineages. We found that lactate and diacetate do synergistically inhibit the growth of *L. monocytogenes* and the response to these GI are similar for the selected strains from the two lineages tested.

Materials and Methods

Strain selection. A total of 13 *L. monocytogenes* isolates (**Table 1**) were selected for growth in Brain Heart Infusion (BHI; Difco, Detroit, MI) broth treated with growth inhibitors. These strains include 7 that belong to lineage I and 6 that belong to lineage II. Among these strains, there is one representative of each of the 10 unique ribotypes isolated from smoked seafood *(15)*, as well as representatives of serotypes commonly involved in foodborne outbreaks (serotypes 4b, 1/2a, and 1/2b). Strains of serotype 1/2c as well as strains belonging to lineage III were not included in this strain set, as these subtypes are rarely associated with human disease *(33, 52)*.

Growth Media and Conditions. Growth experiments were designed around a two-stage protocol to approximate the transition from pre-growth in a nutrient-limited (e.g. food processing) environment to growth in a nutrient-rich (e.g. food product) environment treated with GI and held at refrigeration temperature. The medium for the pre-growth was a chemically defined, minimal medium specific to *L. monocytogenes (2)* formulated with 25mM glucose as the carbon source. Media for the experimental growth tests was based on BHI media modified to have 4.65% water phase [w.p.] NaCl and pH 6.1, the levels present in commercially processed cold smoked salmon (48), and treated with diacetate and/or lactate at levels that would slow but not entirely prevent growth over the monitored time. BHI was modified to make

Table 1. Subtype and source information for *Listeria monocytogenes* strains used in this study.

Strain	Ribotype	Lineage	Serotype	Source	Reference
FSL C1-122	1038B	I	4b	Human, sporadic	*(11, 34)*
FSL F2-693	1042B	I	1/2b	Human, sporadic	*(35)*
FSL R2-154	1042C	I	1/2b	Smoked seafood	*(14, 15)*
FSL R2-182	1043A	I	1/2b	Smoked seafood	*(14, 15)*
FSL J2-064	1052A	I	1/2b	Bovine	*(6, 11)*
FSL J1-175	1042A	I	1/2b	Water	_[a]
FSL F6-366	1044A	I	4b	RTE Meat	*(29)*
FSL F2-216	1039A	II	1/2a	Human, epidemic	*(15, 36)*
FSL J2-003	1039C	II	1/2a	Bovine	*(6)*
FSL F2-032	1045B	II	1/2a	Smoked whitefish	*(35)*
FSL F2-515	1062A	II	1/2a	RTE meat	*(35)*
FSL F2-237	1062D	II	1/2a	Smoked salmon	*(35)*
FSL R2-559	1053A	II	1/2a	RTE meat	*(29)*

[a]-, no data

this work more relevant to a particular food product, cold smoked salmon, though it is not intended as a challenge study, and organic acid levels were not intended to entirely prevent growth so that we could fully parameterize the logistic bacterial growth model (see 'Model Development and Statistical Analysis') used for analysis of the measured growth data.

Treatment media were formulated on a water phase basis, by weight, where: % water phase [% w.p.] = g dry component / (g dry component + g water). For example, to formulate a solution of BHI with 4.65% w.p. NaCl, first a standard BHI solution was made by adding 37 g BHI powder to 1L (1 kg) water noting that BHI contains 5g NaCl per 37 g powder, then additional NaCl (43.8 g) was added to the solution so the final water phase NaCl (5g + 43.8g / (5g + 43.8g + 1000 g) was 4.65%. The treatment media described below were formulated similarly, adding commercial compounds, by weight, to achieve the desired levels while accounting for the relative percentages of organic acid, water, and other compounds in each commercial stock.

The four treatment media (Table 2) were formulated with (i) no added inhibitors as control [CTRL], (ii) 0.14% w.p. sodium diacetate [SDA] (Macco Organiques Inc., Valleyfield, Quebec, Canada), (iii) 2% w.p. potassium lactate [PL] (PURASAL Hi Pure P-Plus, PURAC America, Inc., Lincolnshire, IL), or (iv) a commercial combination of both inhibitors at the same levels (2% PL + 0.14% SDA) [PLSDA] (PURASAL OptiForm PD-Plus, PURAC America, Inc.). We chose to use potassium lactate instead of the other commonly available alternative, sodium lactate, to be consistent with other recent publications of interest to the smoked fish industry that evaluate potassium lactate, e.g. *(50)*, and because recent efforts at sodium reduction *(26)* in many foods make the potassium salt more attractive than the sodium salt.

Table 2. Formulations for BHI broth media treated with GI.

Treatment	BHI (%wp)[a]	NaCl (%wp)	Diacetate (%wp)	Lactate (%wp)
CTRL	3.70	4.65	-[b]	-
SDA	3.70	4.65	0.14	-
PL	3.70	4.65	-	2.00
PLSDA	3.70	4.65	0.14	2.00

[a] %wp, percent water phase [g of dry component / (g of dry component + g of water)].

[b] -, not included.

It is generally believed that organic acids are biologically active in their undissociated form, the concentration of which can be calculated using the Henderson-Hasselbalch equation: pH = pKa + log([dissociated form]/[undissociated form]) where pH is the pH of the solution, [] indicate the molar concentration of either form of the organic acid, and the pKas are constants for lactate (3.79) and diacetate (4.79, the pKa for acetate, the base anion of diacetate) *(21)*. Using the Henderson-Hasselbalch equation, the percentage of undissociated lactate and acetate in a pH 6.1 solution was calculated and multiplied by the concentration (mol total organic acid / kg of solution) of lactate and diacetate in the treatment solutions, giving undissociated acid concentrations of 0.71 and 0.39 mM/kg_{total} for solutions containing lactate and/or diacetate, respectively. For the remainder of this paper we refer to the water phase percentages for consistency with recent work evaluating these growth inhibitors in food products.

For growth prior to inoculation in media with growth inhibitors, isolates were streaked from frozen stocks of BHI culture maintained at -80°C in 15% glycerol onto BHI agar plates, and incubated at 37°C for at least 24 h. Single colonies of each strain were inoculated into 5 ml BHI (in 16 mm tubes) which were incubated overnight at 37°C with constant shaking at 230 rpm; Series 25 Incubator, New Brunswick Scientific, Edison, NY). After 16-20 h, 50 µl of BHI culture were used to inoculate 5 ml of sterile *L. monocytogenes* chemically defined medium *(2)* pre-warmed to, and incubated statically at, 16°C. After growth to log-phase, defined as OD_{480} = 0.4 (~24h), 50 µl of culture were transferred to another 5 ml chemically defined medium at 16°C and grown to early stationary phase which was reached after 72 h (Figure 1).

After growth in defined medium, stationary phase cells were used to inoculate treatment media at ~2 x 10^3 CFU/ml. Treatment media was formulated according to Table 2, adjusted to a pH of 6.1 using 6 N HCl and 5 N NaOH, as needed, sterilized

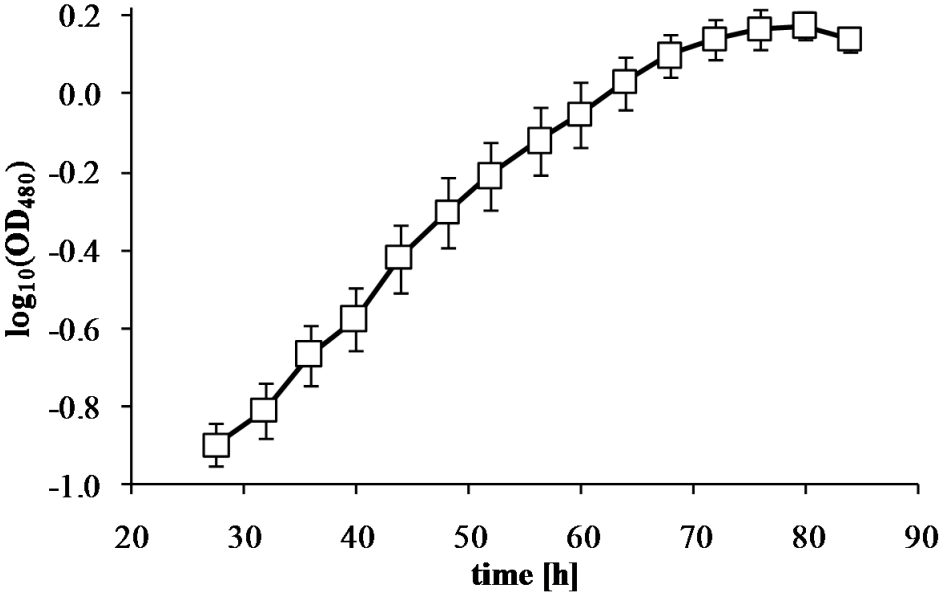

Figure 1. Growth of *Listeria monocytogenes* in defined, minimal medium at 16°C. Each point represents the average $\log(OD_{480})$ and standard deviation from 13 strains. Readings outside the linear range of the spectrophotometers [$\log(OD_{480}) \leq 1.0$] were excluded.

by autoclaving and then stored at 4°C. Before each test, fresh media were prepared; 75 ml for each treatment and strain were aseptically aliquoted into sterile, 300 ml Erlenmeyer shake flasks with metal caps (Bellco Glass Co, Vineland, NJ) and chilled to 7°C. Cultures were diluted 1:1,000 in phosphate buffered saline and 75 µl of the dilution were used to inoculate each of the treatment flasks filled with 75 ml treatment media for a final dilution of $1:10^6$. Samples of each treatment flask were reserved for plating and the flasks were stored, without aeration, at 7°C (the temperature that at least 90% of consumer refrigerators are at or below *(3)*) in a low-temperature incubator (VWR Model 2020, West Chester, PA) for the duration of the growth period. To better characterize lag phase, cell density was measured every day from day 2 until growth began; subsequently, cell densities were determined every other day until cultures reached stationary phase. For all samples, cultures were serially diluted in phosphate buffered saline, 50 µl of the appropriate dilution was plated in duplicate on BHI agar using an Autoplate 4000 and colonies were counted with a Q-Count (Spiral Biotech, Inc., Norwood MA) after incubation at 37°C for at least 24 h. Growth for each strain in each treatment was measured in duplicate.

Growth Model and Statistical Analysis. The four-factor modified logistic growth model described by Baranyi and Roberts *(5)* was used to calculate a lag phase (λ), maximum growth rate (μ_{max}) initial cell density (N_0) and maximum cell density (N_{max}) for each strain for each GI treatment. Regression was carried out using the NLStools package (v0.0-5) in R v2.6.2. Differences between treatments were analyzed using a separate fixed effect ANOVA for each parameter. This design had 4 factors: lactate, diacetate, evolutionary lineage, and biological replicate, each with two levels. The linear model used for the ANOVA was *Y* (the growth parameter) = lactate | diacetate | lineage + replicate + *E* (error), where "|" indicates a full factorial model of the grouped parameters. The relative explanatory contribution of each parameter was

quantified by calculating the percentage of the total variation (squared error) in the data accounted for by each parameter, i.e. sum squared error for each parameter / total sum squared error for the model. Comparisons of interest, specifically the diacetate-lactate and diacetate-lactate-lineage interactions, were compared using Tukey's correction for multiple comparisons. Additionally, multivariate correlation coefficients were calculated to quantify the linear relationship between the regression parameters. Correlations were calculated after (i) grouping the parameters as one data set, i.e. calculating the overall correlation in the data including the variability caused by growth inhibitor treatment, and (ii) grouping the parameters by growth inhibitor treatment, i.e. calculating the correlated nature of parameters within a specific treatment separate from the potentially correlated nature of the effects of the treatments themselves. ANOVAs, multiple comparisons and multivariate correlation calculations were performed in JMP (v. 7.0, SAS Institute Inc., Cary, NC).

Monte Carlo simulations. The effect of PL and SDA GI on *L. monocytogenes* growth was studied using Monte Carlo simulation *(32)* to predict growth to two different endpoints based on modified logistic growth model parameters sampled from distributions fitted to the experimental growth parameters. The two endpoints used were the time (in days) for 1-log growth from the initial cell density, and the time (in days) for growth to 99% of the final cell density. Simulations were performed using the @RISK (v. 4.5, Palisade Corp., Ithaca, NY) software package integrated into Microsoft Excel 2003 (Microsoft Corp., Redmond, WA).

First, @RISK was used to fit 15 different statistical distributions (BetaGeneral, Exponential , ExtremeValue, Inverse Gaussian, Logistic, Log-Logistic, Log Normal, Normal, Pareto, Pearson 5 parameter, Pearson 6 Parameter, Triangle, Uniform, and Wiebull) to the experimental data (13 strains, 2 reps, n = 26) for each of the 16 unique regression parameters (λ, μ_{max}, N_0 and N_{max} for each of the 4 growth inhibitor

treatments), and goodness of fit evaluated with a chi-square test. The normal distribution had the largest p-value averaged over all 16 regression parameter fits, therefore was the best fit, and was selected for the Monte Carlo simulations. Next, sampled growth parameters for each treatment were used to predict the times required for colony count to increase by 1-log [t where $N(t) = N_0 + 1$ where $N(t)$ and N_0 are measured in log(CFU per milliliter)] and for colony counts to reach 99% of the final density [t where $N(t) = N_0 + 0.99(N_{max} + N_0)$]. For each iteration of the Monte Carlo simulation, 16 new regression parameters were sampled as correlated, normally distributed inputs, where the correlations between parameters for each treatment are the calculated correlation coefficients for the experimental data grouped by treatment, and the normal distribution is defined by the experimental mean and standard deviation for each treatment. Then, 8 new growth times, one for each treatment and endpoint combination, calculated from the sampled parameters were recorded as outputs. The simulation was run for 5000 iterations using Latin Hypercube sampling for the input parameters. Output data were analyzed by median value (50^{th} percentile), inner quartile range (25^{th} – 75^{th} percentile) and 95% confidence intervals for growth time (2.5^{th} - 97.5^{th} percentile) which were found by sorting the results from the 5000 iterations by time and reading the appropriate values.

Results

Strains from evolutionary lineage I and II respond similarly to GI treatments. To determine if the evolutionary lineages of L. monocytogenes responded differently to the GI treatments, we compared growth parameters for the four GI treatments between the lineages. Growth curves for all 13 strains and four GI treatments (CTRL, SDA, PL, and PLSDA) were measured in duplicate, and modified logistic growth parameters (λ, μ_{max}, N_0 and N_{max}) were fit to each individual curve (n = 104). Of all the ANOVA model effects containing lineage (lineage alone and all its crosses), only

the main effect of lineage on λ was significant (P = 0.0499). Overall, lineage II strains have a slightly longer average lag time, estimated to be 0.32 day longer than the average lag time for lineage I strains. Although lineage II strains trend towards having a longer lag times, lag time differences between evolutionary lineage were not significant for a given treatment (Table 3). All other growth parameters had virtually indistinguishable values when the effect of GI treatments was analyzed by lineage. Because evolutionary lineage did not have a major effect on growth, all further analyses group the data from both lineages together.

To determine the contribution of strain to variation in growth parameters, an additional ANOVA model with a strain predictor nested within lineage was used to analyze the growth parameters (full data not shown). In this analysis, strain had a significant (p = 0.006), but small effect on μ_{max}, explaining 5.9% of the total variation of the new model. Strain had a significant (P < 0.001), and greater, effect on N_o and N_{max}, explaining 50% and 22% of the variation, respectively. The variation in N_0 due to strain reflects the variability in final cell densities in chemically defined media (Fig. 1) before inoculation into the treatment media. The significant effect of strain on N_{max} indicates that different strains may grow to slightly different final densities in BHI. For both N_o and N_{max}, average differences in cell densities were less than 0.5 log(CFU/ml).

PL and PLSDA significantly increase lag phase and reduce maximum growth rate. The comparisons between growth inhibitor treatments are summarized in Table 4 and the individual parameters are available in Table A1. PL alone significantly (P < 0.001) extended λ from 2.6 to 3.8 days compared to CTRL (Table 4). SDA alone had no effect on λ (P = 0.79), but when added to PL, significantly (P < 0.001) increased λ to 6.6 days, an extension of almost 3 days

Table 3. Lineage effects on growth parameters for *Listeria monocytogenes* at 7°C[a]

Treatment	Lineage	λ(days)	μ_{max} [log(CFU/ml)/day]	N_0 [log(CFU/ml)]	N_{max} [log(CFU/ml)]
CTRL	I	2.57 ± 0.37 A[b]	0.54 ± 0.05 AB	3.40 ± 0.10 A	8.56 ± 0.21 BC
	II	2.66 ± 0.43 A	0.54 ± 0.07 A	3.35 ± 0.10 A	8.64 ± 0.22 AB
SDA	I	2.54 ± 0.39 A	0.51 ± 0.03 AB	3.40 ± 0.14 A	8.69 ± 0.16 AB
	II	2.81 ± 0.25 AB	0.52 ± 0.02 AB	3.38 ± 0.13 A	8.81 ± 0.16 A
PL	I	3.73 ± 0.37 BC	0.49 ± 0.04 AB	3.42 ± 0.08 A	8.31 ± 0.16 D
	II	3.92 ± 0.67 C	0.49 ± 0.04 B	3.40 ± 0.07 A	8.33 ± 0.22 CD
PLSDA	I	6.29 ± 1.36 D	0.34 ± 0.04 C	3.43 ± 0.09 A	8.21 ± 0.15 D
	II	7.02 ± 1.72 D	0.33 ± 0.06 C	3.40 ± 0.09 A	8.22 ± 0.29 D

[a] Results are summarized by mean ± standard deviation for seven lineage I and six lineage II strains tested in duplicate.

[b] Means within a given column with the same letter are not statistically different from each other (overall $\alpha = 0.05$, Tukey's correction)

Table 4. GI treatments affect growth parameters for *Listeria monocytogenes* at 7°C[a]

Treatment	λ (days)	μ_{max} [log(CFU/ml)/day]	N_0 [log(CFU/ml)]	N_{max} [log(CFU/ml)]
CTRL	2.61 ± 0.39 A[b]	0.54 ± 0.06 A	3.38 ± 0.10 A	8.60 ± 0.21 A
SDA	2.68 ± 0.35 A	0.52 ± 0.03 AB	3.39 ± 0.13 A	8.75 ± 0.17 B
PL	3.82 ± 0.53 B	0.49 ± 0.04 B	3.41 ± 0.08 A	8.32 ± 0.18 C
PLSDA	6.63 ± 1.55 C	0.34 ± 0.05 C	3.41 ± 0.09 A	8.21 ± 0.22 C

[a] Results are summarized by mean ± standard deviation for 13 strains tested in duplicate (n=26 per treatment).

[b] Means within a given column with the same letter are not statistically different from each other (overall α=0.05, Tukey's correction)

compared to PL alone. PL significantly ($P < 0.001$) lowered μ_{max} compared to the control, from 0.54 to 0.49 log(CFU/ml)/day. The PLSDA treatment further lowered ($P < 0.001$) μ_{max} to 0.34 log(CFU/ml)/day. Signficant differences in N_0 were not observed between the 4 treatments. N_{max} was signficantly ($P < 0.001$) lower in both treatments containing PL (8.32 log(CFU/ml) for PL and 8.21 log(CFU/ml) for PLSDA) compared to the other treatments (8.60 log(CFU/ml) for CTRL and 8.75 log(CFU/ml) for SDA), though the difference of less than 0.5 log(CFU/ml) may not be biologically relevant.

Further, the growth parameters λ, μ_{max} and N_{max} were significantly ($P < 0.001$, by pairwise comparison) correlated in the full data set, as suggested by the means in Table 4, with λ inversely correlated to μ_{max} (Pearson's correlation coefficient was -0.88) and N_{max} (-0.72) and μ_{max} directly correlated to N_{max} (0.70). The trends in the correlations between parameters grouped by growth inhibitor treatment were similar (e.g. -0.48, -0.53, and 0.78 for λ to μ_{max}, λ to N_{max}, and μ_{max} to N_{max} in the CTRL treatment), though generally the correlations were weaker and often non-significant (Table A2).

Synergistic effects of PL and SDA on λ and μmax. Beyond the comparisons of treatment means, ANOVA showed a significant interaction between diacetate and lactate for λ ($P < 0.0001$), μ_{max} ($P < 0.0001$) and N_{max} ($P = 0.002$) (Table 5), indicating that these GI act synergistically in this system. The synergistic effect of PL and SDA on λ is evident, as SDA alone was ineffective at increasing λ, yet the combination of PLSDA led to an average 2.5 times longer λ compared to CTRL (Figure 2A). The combination of PLSDA led to an average 1.6 times slower μ_{max} compared to CTRL, while SDA alone and PL alone had minimal effects on μ_{max} (Figure 2B). Whether

Table 5. Summary of ANOVA for effects of GI treatments and lineage on growth parameters.

	Type III ANOVA *P* values for:				**Percent of total squared error for[a]:**			
Model Effect	**λ**	**μ_{max}**	**N_0**	**N_{max}**	**λ**	**μ_{max}**	**N_0**	**N_{max}**
ANOVA overall	*<0.001*[b]	*<0.001*	0.572	*<0.001*	NA	NA	NA	NA
SDA	*<0.001*	*<0.001*	0.706	0.555	15.43	22.78	0.14	0.10
PL	*<0.001*	*<0.001*	0.155	*<0.001*	49.50	41.20	2.02	31.83
SDA x PL	*<0.001*	*<0.001*	0.853	*0.002*	14.12	12.85	0.03	3.02
Lineage	*0.0499*	0.885	0.140	0.132	0.78	0.00	2.17	0.66
SDA x lineage	0.258	0.947	0.771	0.796	0.25	0.00	0.08	0.02
PL x lineage	0.425	0.435	0.901	0.281	0.13	0.14	0.02	0.34
SDA x PL x lineage	0.622	0.908	0.783	0.740	0.05	0.00	0.07	0.03
Replicate	*0.022*	*0.011*	0.147	0.279	1.06	1.52	2.10	0.34
Error	NA	NA	NA	NA	18.68	21.51	93.35	63.65

[a] SSE_{effect} / SSE_{total} x 100.

[b] Significant ($P < 0.05$) effects are in italics.

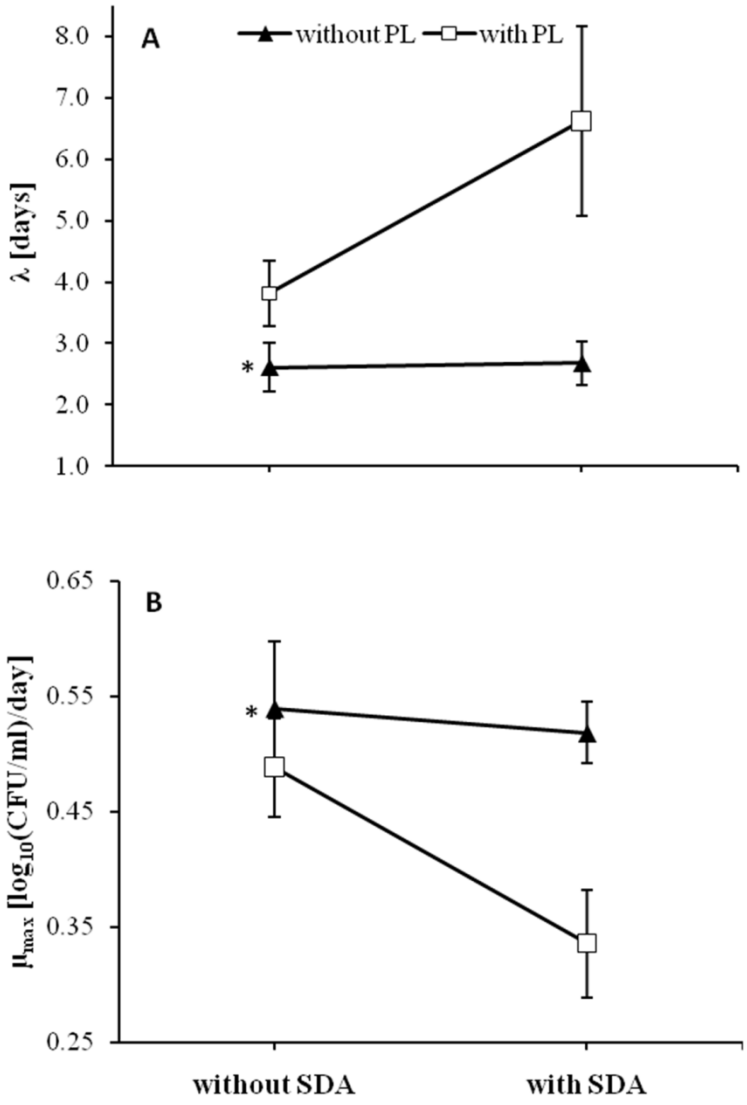

Figure 2. Changes in λ (A) and μ_{max} (B) are dependent on the presence or absence of PL and SDA in the growth medium. □, with PL treatment, ▲, without PL treatment. Points are means of growth parameters for each inhibitor treatment with error bars of 1 standard deviation (n = 26 per treatment). The control treatment is marked with an asterisk.

statistical interaction implies synergy for N_{max} is unclear because of the conflicting action of SDA. Alone, SDA significantly ($P < 0.01$), though only slightly, increases N_{max} compared to CTRL, but when used in combination with PL the action is reversed, with a non-significant ($P = 0.064$) reduction in N_{max} for PLSDA as compared with PL.

PL, SDA and PLSDA have the largest effect on variance of growth parameters. The relative contributions of each predictor to the overall variation in the experimental data was calculated (Table 5) was percentage of total squared error. For λ and μ_{max} the largest source of variation was PL, with 49 and 41% of the total for λ and μ_{max}, respectively. This indicates that the main contribution to variation in λ and μ_{max} was due to the presence or absence of lactate in the growth medium. The contributions of SDA and the PL-SDA interaction to variance in λ and μ_{max} was half or less than the effect of PL, respectively 15 and 14% for λ and 23 and 13% for μ_{max}. These relative percentages for λ and μ_{max} were similar to the unexplained variation in the data, represented by the error with 19 and 22%, respectively. Biological variation also had a significant, though minor, effect for these parameters, indicated by the small contribution of the "replicate" predictor to the variation (1.1 and 1.5% for λ and μ_{max}, respectively). For N_{max}, only error, PL and the SDA-PL interaction had significant explanatory power, corresponding to 64, 32 and 3.0% of the variance.

Treatment with the combination of lactate and diacetate slows L. monocytogenes growth to nominal endpoints. Monte Carlo simulations were used to predict the effect of GI on the time it takes for *L. monocytogenes* to increase colony counts (i) by 1 log and (ii) to within 99% of its maximum density. Of the 15 distributions tested, only 3-logistic, normal and triangle- were successfully fitted to all 16 unique growth parameters. Average *P* values to reject the chi-square test for goodness of fit were 0.636, 0.638 and 0.618 for the logistic, normal and triangle distributions, respectively. All 16 unique growth parameters (λ, μ_{max}, N_0, and N_{max} for

each GI treatment) were therefore modeled with normal distributions for the Monte Carlo simulations, using the experimental average and standard deviation values shown in Table 4 and the calculated correlations for the regression parameters grouped by treatment (Table A2).

Results from the Monte Carlo simulations for the time for colony counts to increase by 1log and to 99% of maximum density suggest that while individual GI might not be effective in extending the time to reach these endpoints, the combination treatment may be effective. The estimated times for growth to each endpoint do not significantly differ for single growth inhibitor treatments compared to CTRL (Figure 3) and the median times showed only very slight increases, 5.79 and 15.7 days for PL and 4.52 and 14.7 days for SDA compared to 4.40 and 14.0 days for CTRL, for times to increase by 1 log and to 99% of final density, respectively. Conversely, the PLSDA treatment was predicted to significantly (95% confidence) increase the time required for colony counts to increase to 99% of final density compared to CTRL and both SDA and PL inhibitor treatments, to a median 23.8 days (95% confidence interval of 19.0 to 30.4 days), a >50% increase in median time. Similarly, PLSDA treatment was predicted to significantly increase the time required for colony counts to increase by 1log compared to CTRL and SDA treatment, to a median of 9.52 days (95% confidence interval of 6.31 to 12.8 days), also a >50% increase in median time. Although the median predicted time for colony counts to increase by 1 log increased by over 50% for the PLSDA treatment compared to PL, the 95% confidence intervals for each treatment overlap. The greater uncertainty in the PLSDA predictions may be partially attributed to the increased variability in the observed lag times for that treatment, evidenced by standard deviation of λ at least threefold greater than in the other treatments (Table 4)

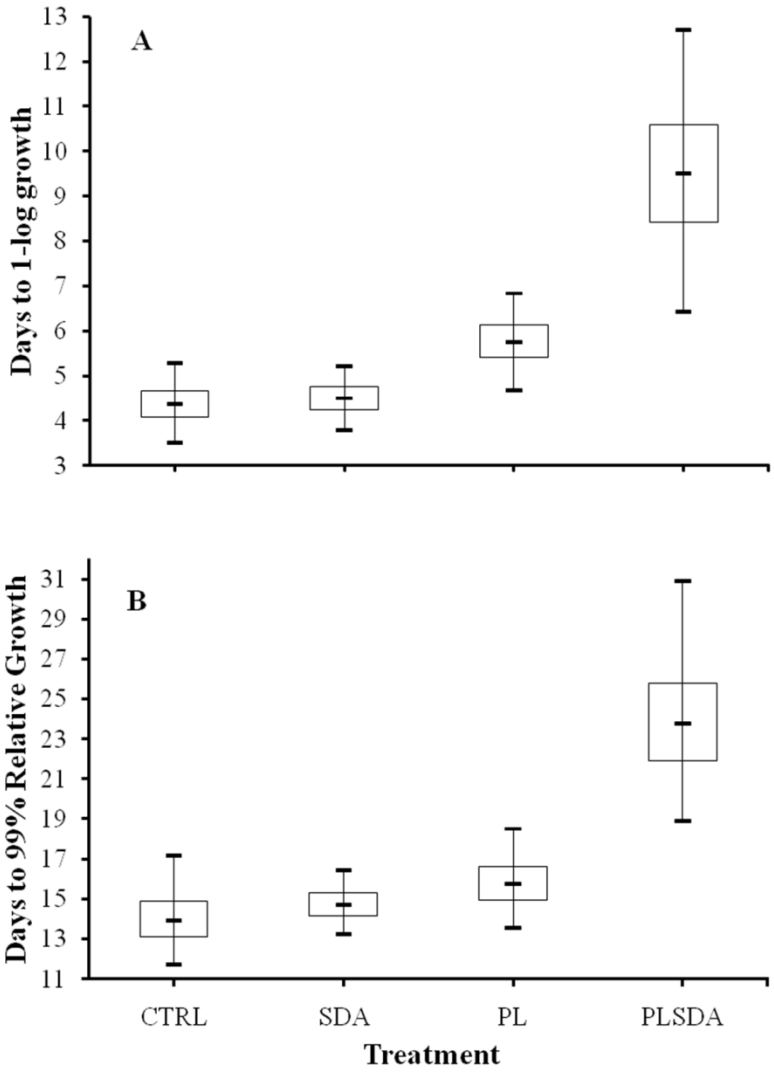

Figure 3. Diacetate and lactate synergistically extend the time predicted by Monte Carlo simulations required for *Listeria monocytogenes* outgrowth. (A) Time required for colony counts to increase by 1 log from predicted N_0 ($N_{1\ log} = N_0 + 1$). (B) Time required for colony counts to reach 99% of the difference between N_{max} and N_0 [$N_{99\%} = N_0 + 0.99\ (N_{max} - N_0)$]. Median line is the 50th quantile, box is drawn at the 25th and 75th quantile, and the whiskers are drawn at the 97.5th and 2.5th quantile from 5,000 iterations.

Discussion

This study investigated the effects of sodium diacetate, potassium lactate, and their combination on the growth of strains representing two evolutionary lineages of *L. monocytogenes* at 7°C in broth media adjusted to the water phase salt and pH of cold smoked salmon. *L. monocytogenes* counts over time were used to generate modified logistic model parameters characterizing λ, μ_{max}, N_0, and N_{max}. Statistical analysis of these growth parameters showed that PL and SDA synergistically interact to extend λ and lower μ_{max}, while N_{max} was slightly reduced by lactate treatment. Monte Carlo simulations further suggest synergy between diacetate and lactate on slowing growth to nominal endpoints (i.e. time for colony counts to increase by 1-log and to 99% of maximum density).

The effect of SDA and PL treatments can be separated into effects on individual growth parameters. By evaluating changes in growth rate parameters, these two growth inhibitors were shown to affect three distinct elements of a bacterial growth curve, each of which can be independently analyzed and used to assess growth inhibitor efficacy. Decomposition of the elements of a growth curve has been used to evaluate the effect of other hurdles, e.g. PLSDA, growth temperature, and freezing stress *(54)* or PLSDA, growth temperature, and pH *(1)*, on the lag time and specific growth rate of *L. monocytogenes*.

After fitting logistic model parameters to experimental growth curves from CTRL, SDA, PL and PLSDA treatments, our statistical analysis of the parameters showed that SDA and PL caused different effects on bacterial growth. Lactate alone increased λ, from 2.6 to 3.8 days, and lowered μ_{max}, from 0.54 to 0.49 log(CFU/ml)/day, whereas SDA alone showed no significant effect on either parameter. In the combined treatment, PLSDA, both λ and μ_{max} were further lowered compared to PL alone. Studies that have evaluated the effects of a range of PL and

SDA levels on lag phase and specific growth rate in broth at different temperatures and pH *(1)*, on the predicated growth-no growth boundary in various cold-smoked seafood products *(25)*, and on storage temperature *(54)*, suggest that the levels of GI used in our study, 2.0% potassium lactate and 0.14% sodium diacetate, fall at a transition between slowing and fully inhibiting growth. Of particular relevance, Vogel et al. *(50)* used levels of PL (2.0% w.p.) and SDA (0.14% w.p.) identical to those used here, and reported moderately slower growth of *L. monocytogenes* in cold smoked salmon juice at 10°C as compared to our findings (in modified BHI broth) that individual inhibitors have minimal effect. In contrast to our results, which showed very limited effects of the GI treatments on N_{max}, Vogel et al. found the combination of the two inhibitors completely prevented growth for 28 days in salmon juice and minced cold-smoked salmon at 10°C. At these transition levels, differences in the complex nature of broth and food systems may influence the growth boundary, e.g. chemical changes in salmon during smoking such as the incorporation of phenolic compounds, may add additional hurdles to growth that lead to the differences between our results and those in Vogel et al *(50)*. Hence, our data do not indicate that combination treatments cannot be used to formulate RTE products that do not allow for growth, but rather suggest that GI combinations need to be optimized for specific food matrices.

N_{max} was affected by PL, where treatments containing potassium lactate had a reduced $N_{max,}$ although only by ~0.5 log(CFU/ml). Other studies have shown inconsistent effects of PL and SDA on N_{max}, likely due to differences in temperature and food matrix. One study showed a slightly lower (~0.5 log) maximum density following lactate treatment in refrigerated bologna *(41)*; another found that maximum population density decreased from 8.5 to 7.1 log(CFU/g) at 9.1°C following increasing PLSDA treatments but not higher storage temperatures *(19)*; and a different study

showed that growth in brine injected, minced, cold smoked salmon treated with combinations of PLSDA showed no difference in final density over 28 day at 10°C, but over ~40 days at 10°C growth on cold smoked salmon injected with 2.1% PL and 0.12% SDA stopped after ~2-log increase *(50)*. These studies suggest PL may cause a slight reduction in maximum cell density in some food systems, though further work is needed to conclusively demonstrate such an effect.

SDA and PL synergistically extend lag phase and reduce maximum growth rate. Our data show that PL and SDA interact in a statistically significant manner to increase λ and reduce μ_{max}, indicating a synergistic action of these growth inhibitors when used in combination *(39)*. The synergistic effects of lactate and diacetate are consistent with other studies that were not necessarily designed to test this relationship. For example, Neetoo et al. *(28)* found, during growth at 35°C in tryptic soy agar with 0.6% yeast extract, that while sodium lactate and SDA did not prevent growth at half their MICs (2.8 and 0.11% [wt/wt], respectively), the combination did prevent growth. Additionally, sodium lactate at 2.4 or 4.8% and SDA at 0.125% allowed growth, the binary combinations (sodium lactate 2.4 or 4.8% with SDA 0.25%) prevented growth.

One potential application of the demonstrated synergy is to develop predicative secondary models that incorporate interactions between GI for more accurate prediction of growth responses. Various model have been proposed including (i) a response surface modeling lag phase duration and growth rate of *L. monocytogenes* as a function of temperature, lactate concentration and diacetate concentration where the synergy was explicitly quantified by a parameter scaling the lactate X diacetate interaction term *(19)* (ii)a model for the maximum growth rate of *L. monocytogenes* incorporating many environmental parameters that included an interactions effect term where the individual effects of lactate and diacetate were multiplied *(25),* and

predicted the growth-no growth boundary of *L. monocytogenes* having a non-linear, concave up, relationship between lactate and diacetate levels; and (iii) a multiplicative model for growth rate based on fractions of cardinal growth parameters that incorporates interactions between environmental parameters and inhibitory compounds *(4)*, which assumes multiplicity of individual inhibitory effects. Contrary to these claims to model interactions, Lambert and Bidlas *(22)* argue that apparent interactions between combined growth inhibitors are actually the result of independent inhibitor effects, e.g., modeling interactions with multiplicative terms but not additional empirical parameters (as in *(4)*) indicates a more complex independent effect, not interactions. Regardless of the specific details, accurate modeling of the synergistic effects of growth inhibitor combinations is a critical step in optimizing treatment levels for food products.

Strains from two evolutionary lineages respond similarly to growth inhibitor treatment when grown at 7°C in broth**.** Our data has shown that strains representing *L. monocytogenes* lineage I or II do not have significantly different mean values for any of the four growth parameters during growth in media treated with organic acid GI, although ANOVA analysis did show lineage II had slightly longer lag times overall. Vogel et al. *(50)* found that two strains of different lineages and serotypes (lineage I serotype 4, lineage II serotype 1/2 *(49)*) isolated from smoked seafood processors had similar growth curves in minced cold smoked salmon treated with the same combination of lactate and diacetate (2% PL and 0.14% SDA). In contrast, De Jesus and Whiting *(10)* found that strains representing lineage II had significantly shorter mean lag-time durations than strains representing either lineage I or III when grown at 5°C in BHI at pH 6.5 with 0.1 M lactate and Lianou et al. *(23)* found that one lineage II strain had a higher maximum growth rate in tryptic soy broth with 0.6% yeast extract at 4°C than three other lineage I strains and one lineage III strain. The

ambiguity in the literature suggests that the effect of genetic lineage on growth at low temperatures in the presence of organic acids may be minor and further studies will need to be designed to specifically isolate lineage effects from confounding factors such as serotype or source.

The minimal effect of lineage on low-temperature growth observed here contrasts with research that has shown virulence, metabolic, and ecological differences between lineages. Lineage I strains have a predicted infectious dose about 5 orders of magnitude higher than lineage II strains *(7)*. Lineage I isolates are overrepresented among human clinical isolates, while lineage II isolates are overrepresented among environmental and food isolates *(15)*. It seems that although evolutionary lineage does affect some aspects of *L. monocytogenes* physiology, those differences may not be highly relevant to the ability to overcome organic acid GI stresses at low temperature under the conditions used here.

Monte Carlo simulations may be useful predictors of GI treatment efficacy. The preceding analyses of the effect of GI on growth parameters are useful analytical tools but do not directly address the fundamental question of whether or not GI, alone or in combination, lead to a reduction in *L. monocytogenes* levels at the time of consumption, therefore reducing the risk of human listeriosis. Such a question is better addressed by risk assessments that use both scientific knowledge and uncertainty to come to a conclusion *(45)*. Risk assessments are useful because they quantify the uncertainty in growth responses based on data from many strains and avoid extrapolating from growth data based on a few, or even a single, strain. Given that members of the scientific community *(18, 46)*, as well as some U.S. *(44)* and EU *(9)* regulatory agencies, are considering the principle that RTE foods contain less than 100 CFU/g of *L. monocytogenes* at the end of their shelf life are acceptable for human

consumption, accurate assessment of growth throughout a product's shelf life will become essential to product safety.

We used our experimentally measured growth parameters as the input for Monte Carlo simulations to probe two questions, the time required for *L. monocytogenes* to increase in colony count (i) by 1 log(CFU/ml) and, (ii) to 99% of its maximum density, based on the distribution of growth parameters measured experimentally. Although statistical analysis of growth parameters showed that individually PL would have significant effects on λ and μ_{max}, the Monte Carlo simulations show more uncertainty, and do not support the conclusion that individual GI, at the levels used in this study, would have any effect on outgrowth times in broth. Yet, the 95% confidence intervals of the PLSDA simulations do show that the combined treatment of BHI broth with 0.14% SDA and 2% PL will extend the time required for growth to both endpoints compared to the control. These results imply that although the individual growth inhibitors tested here are not strong enough to slow growth alone, the synergies proven in deconstructive analyses do create strong enough inhibition that the combination treatment would effectively slow growth of *L. monocytogenes* in broth to increases of 1-log colony counts and to within 99% of the final density. In the future, this approach, along with optimization of GI levels for specific foods, could be used to help predict acceptable shelf life, taking in consideration appropriate public health goals, such as Healthy People 2010 *(43)*.

In conclusion, by proving statistical interaction between GI treatments on growth parameters, we found that PL and SDA act synergistically to extend the lag phase and reduce the maximum growth rate of *L. monocytogenes* grown in BHI broth media at 7°C, modified for physiological relevance to cold smoked salmon (4.65% w.p. salt at pH 6.1). Specifically, 2.0% w.p.PL alone increased lag time and reduced the maximum growth rate, and further growth inhibition was gained by adding 0.14%

SDA. Monte Carlo simulations based on these data predict that while both PL and SDA used at levels tested here are ineffective at reducing growth of *L. monocytogenes*, their use in combination will significantly extend the time needed for this pathogen to grow to potentially dangerous levels in broth media at refrigeration temperature. Future challenge studies in real food products can utilize the synergistic action of potassium lactate and sodium diacetate to develop efficient, growth inhibiting, product formulations at minimal cost to processors.

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REFERENCES

1. Abou-Zeid, K. A., K. S. Yoon, T. P. Oscar, J. G. Schwarz, F. M. Hashem, and R. C. Whiting. 2007. Survival and growth of *Listeria monocytogenes* in broth as a function of temperature, pH, and potassium lactate and sodium diacetate concentrations. *J. Food Prot.* 70:2620-2625.
2. Amezaga, M.-R., I. Davidson, D. McLaggan, A. Verheul, T. Abee, and I. R. Booth. 1995. The role of peptide metabolism in the growth of *Listeria monocytogenes* ATCC 23074 at high osmolarity. *Microbiol*. 141:41-49.
3. Audits International/FDA. 1999. U. S. food temperature evaluation. Available at: http://www.foodrisk.org/exclusives/audits/downloads/Audits-FDA_temp_study.pdf. Accessed 5 April 2009.
4. Augustin, J.-C., and V. Carlier. 2000. Modelling the growth rate of *Listeria monocytogenes* with a multiplicative type model including interactions between environmental factors. *Int J Food Microbiol*. 56:53-70.
5. Baranyi, J., and T. A. Roberts. 1994. A dynamic approach to predicting bacterial growth in food. *Int. J. Food Microbiol.* 23:277-294.
6. Cai, S., D. Y. Kabuki, A. Y. Kuaye, T. G. Cargioli, M. S. Chung, R. Nielsen, and M. Wiedmann. 2002. Rational design of DNA sequence-based strategies for subtyping *Listeria monocytogenes. J. Clin. Microbiol*. 40:3319-3325.
7. Chen, Y., W. H. Ross, M. J. Gray, M. Wiedmann, R. C. Whiting, and V. N. Scott. 2006. Attributing risk to *Listeria monocytogenes* subgroups: dose response in relation to genetic lineages. *J. Food Prot.* 69:335-344.
8. Chen, Y., W. H. Ross, V. N. Scott, and D. E. Gombas. 2003. *Listeria monocytogenes*: low levels equal low risk. *J. Food Prot.* 66:570-577.
9. Commission of the European Communities. 2008. Guidence Document on *Listeria monocytogenes* shelf-life studies for ready-to-eat foods, under Regulation (EC) No

2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Available at: http://ec.europa.eu/food/food/biosafety/salmonella/microbio_en.htm. Accessed 20 November 2008.

10. De Jesus, A. J., and R. C. Whiting. 2003. Thermal inactivation, growth, and survival studies of *Listeria monocytogenes* strains belonging to three distinct genotypic lineages. *J. Food Prot.* 66:1611-1617.
11. Fugett, E., E. Fortes, C. Nnoka, and M. Wiedmann. 2006. International life sciences institute North America *Listeria monocytogenes* strain collection: development of standard *Listeria monocytogenes* strain sets for research and validation studies. *J. Food. Prot.* 69:2929-2938.
12. Glass, K. A., D. A. Granberg, A. L. Smith, A. M. McNamara, M. Harding, J. Mattias, K. Ladwig, and E. A. Johnson. 2002. Inhibition of *Listeria monocytogenes* by sodium diacetate and sodium lactate on wieners and cooked bratwurst. *J. Food Prot.* 65:116-123.
13. Glass, K. A., L. M. McDonnell, R. C. Rassel, and K. L. Zierke. 2007. Controlling *Listeria monocytogenes* on sliced ham and turkey products using benzoate, propionate, and sorbate. *J. Food Prot.* 70:2306-2312.
14. Gombas, D. E., Y. Chen, R. S. Clavero, and V. N. Scott. 2003. Survey of *Listeria monocytogenes* in ready-to-eat foods. *J. Food Prot.* 66:559-569.
15. Gray, M., R. Zadoks, E. Fortes, B. Dogan, S. Cai, Y. Cen, V. Scott, D. Gombas, K. Boor, and M. Wiedmann. 2004. *Listeria monocytogenes* isolates from foods and humans form distinct but overlapping populations. *Appl. Envrion. Microbiol.* 70:5833-5841.
16. Gutierrez, J., C. Barry-Ryan, and P. Bourke. 2008. The antimicrobial efficacy of plant essential oil combinations and interactions with food ingredients. *Int. J. Food Microbiol.* 124:91-97.

17. Hoffman, A., K. Gall, and M. Wiedmann. 2003. The microbial safety of minimally processed seafood with respect to *Listeria monocytogenes.* p. 53-75. *In* J. Novak, G. Sapers, and V. Juneja. Microbial safety of minimally processed foods. CRC Press.
18. Huss, H. H., L. V. Jorgensen, and B. F. Vogel. 2000. Control options for *Listeria monocytogenes* in seafoods. *Int. J. Food Microbiol.* 62:267-274.
19. Hwang, C. F., and M. L. Tamplin. 2007. Modeling the lag phase and growth rate of *Listeria monocytogenes* in ground ham containing sodium lactate and sodium diacetate at various storage temperatures. 72:246-253.
20. Jahncke, M., R. Collette, D. Hicks, M. Wiedmann, V. N. Scott, and K. Gall. 2004. Treatment options to eliminate or control growth of *Listeria monocytogenes* on raw material and on finished product for the smoked fish Industry. *Food Prot. Trends*. 24:612-619.
21. Lado, B., and A. Yousef. 2007. Characteristics of *Listeria monocytogenes* important to food processors. p. 157-213. *In* E. Ryser, and E. Marth (ed.), *Listeria,* listeriosis, and food safety. CRC Press, Boca Raton, FL.
22. Lambert, R. J. W., and E. Bidlas. 2007. An investigation of the gamma hypothesis: a predictive modelling study of the effect of combined inhibitors (salt, pH and weak acids) on the growth of *Aeromonas hydrophila. Int J Food Microbiol*. 115:12-28.
23. Lianou, A., J. D. Stopforth, Y. Yoon, M. Wiedmann, and J. N. Sofos. 2006. Growth and stress resistance variation in culture broth among *Listeria monocytogenes* strains of various serotypes and origins. *J. Food Prot.* 69:2640-2647.

24. Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe. 1999. Food-related illness and death in the United States. *Emerg. Infect. Dis.* 5:607-625.
25. Mejlholm, O., and P. Dalgaard. 2007. Modeling and predicting the growth boundary of *Listeria monocytogenes* in lightly preserved seafood. *J. Food Prot.* 70:70-84.
26. Nachay, K. 2008. Staying smart about salt. *Food Technol.* 08:26-35.
27. Neetoo, H., M. Ye, and H. Chen. 2008. Potential antimicrobials to control *Listeria monocytogenes* in vacuum-packaged cold-smoked salmon pâté and fillets. *Int. J. Food Microbiol.* 123:220-227.
28. Neetoo, H., M. Ye, H. Chen, R. D. Joerger, D. T. Hicks, and D. G. Hoover. 2008. Use of nisin-coated plastic films to control Listeria monocytogenes on vacuum-packaged cold-smoked salmon. 122:8-15.
29. Nelson, K. E., D. E. Fouts, E. F. Mongodin, J. Ravel, R. T. DeBoy, J. F. Kolonay, D. A. Rasko, S. V. Angiuoli, S. R. Gill, I. T. Paulsen, J. Peterson, O. White, W. C. Nelson, W. Nierman, M. J. Beanan, L. M. Brinkac, S. C. Daugherty, R. J. Dodson, A. S. Durkin, R. Madupu, D. H. Haft, J. Selengut, S. Van Aken, H. Khouri, N. Fedorova, H. Forberger, B. Tran, S. Kathariou, L. D. Wonderling, G. A. Uhlich, D. O. Bayles, J. B. Luchansky, and C. M. Fraser. 2004. Whole genome comparisons of serotype 4b and 1/2a strains of the food-borne pathogen *Listeria monocytogenes* reveal new insights into the core genome components of this species. *Nucleic Acids Res.* 32:2386-2395.
30. Nilsson, L., Y. Chen, M. L. Chikindas, H. H. Huss, L. Gram, and T. J. Montville. 2000. Carbon dioxide and nisin act synergistically on *Listeria monocytogenes.* *Appl. Environ. Microbiol.* 66:769-774.

31. Nykanen, A., K. Weckman, and A. Lapvetelainen. 2000. Synergistic inhibition of *Listeria monocytogenes* on cold-smoked rainbow trout by nisin and sodium lactate. *Int. J. Food Microbiol.* 61:63-72.
32. Poschet, F., A. H. Geeraerd, N. Scheerlinck, B. M. Nicola, and J. F. Van Impe. 2003. Monte Carlo analysis as a tool to incorporate variation on experimental data in predictive microbiology. *Food Microbiol*. 20:285-295.
33. Roberts, A., K. Nightingale, G. Jeffers, E. Fortes, J. M. Kongo, and M. Wiedmann. 2006. Genetic and phenotypic characterization of *Listeria monocytogenes* lineage III. *Microbiol.* 152:685-693.
34. Sauders, B. D., E. D. Fortes, D. L. Morse, N. Dumas, J. A. Kiehlbauch, Y. Schukken, J. R. Hibbs, and M. Wiedmann. 2003. Molecular subtyping to detect human listeriosis clusters. *Emerg. Infect. Dis.* 9:672-680.
35. Sauders, B., K. Mangione, C. Vincent, J. Schermerhorn, C. Farchione, N. Dumas, D. Bopp, L. Kornstein, E. Fortes, K. Windham, and M. Wiedmann. 2004. Distribution of *Listeria monocytogenes* molecular subtypes among human and food isolates from New York state shows persistence of human disease-associated *Listeria monocytogenes* strains in retail environments. *J. Food Prot.* 67:1417-1428.
36. Sauders, B. D., Y. Schukken, L. Kornstein, V. Reddy, T. Bannerman, E. Salehi, N. Dumas, B. J. Anderson, J. P. Massey, and M. Wiedmann. 2006. Molecular Epidemiology and Cluster Analysis of Human Listeriosis Cases in Three U.S. States. 69:1680-1689.
37. Schlyter, J., K. A. Glass, J. Loeffelholz, A. Degnan, and J. B. Luchansky. 1993. The effects of diacetate with nitrite, lactate, or pediocin on the viability of *Listeria monocytogenes* in turkey slurries. *Int. J. Food Microbiol.* 19:271-281.

38. Severino, P., O. Dussurget, R. Z. N. Vencio, E. Dumas, P. Garrido, G. Padilla, P. Piveteau, J.-P. Lemaitre, F. Kunst, P. Glaser, and C. Buchrieser. 2007. Comparative transcriptome analysis of *Listeria monocytogenes* strains of the two major lineages reveals differences in virulence, cell wall, and stress response. *Appl. Environ. Microbiol.* 73:6078-6088.
39. Slinker, B. K. 1998. The Statistics of Synergism. 30:723-731.
40. Stack, H. M., C. Hill, and C. G. M. Gahan. 2008. Stress Responses. p. 61-96. *In* D. Liu (ed.), Handbook of *Listeria monocytogenes*. CRC Press, Boca Raton, FL.
41. Thompson, R. L., C. E. Carpenter, S. Martini, and J. R. Broadbent. 2008. Control of *Listeria monocytogenes* in ready-to-eat meats containing sodium levulinate, sodium lactate, or a combination of sodium lactate and sodium diacetate. *J Food Sci*. 73:239-244.
42. Tompkin, R. B. 2002. Control of *Listeria monocytogenes* in the food-processing environment. *J. Food Prot.* 65:709-725.
43. U.S. Department of Health and Human Services. 2000. Healthy people 2010: food safety. Available at: http://www.healthypeople.gov/Document/HTML/Volume1/10Food.htm. Accessed December 2 2009.
44. U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition. 2008. Compliance policy guide - *Listeria monocytogenes*. Available at: http://www.fda.gov/ICECI/ComplianceManuals/CompliancePolicyGuidanceManual/ucm136694.htm. Accessed 10 August 2009.
45. U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition; US Department of Agriculture, Food Safety and Inspection Service; and Centers for Disease Control and Prevention. 2003. Quantitative assessment of relative risk to public health from foodborne *Listeria monocytogenes* among

selected categories of ready-to-eat foods. Available at: http://www.cfsan.fda.gov/~dms/lmr2-su.html Accessed 1 February 2008.

46. van Schothorst, M. 1996. Sampling plans for *Listeria monocytogenes. Food Cont.* 7:203-208.

47. Vazquez-Boland, J. A., M. Kuhn, P. Berche, T. Chakraborty, G. Dominguez-Bernal, W. Goebel, B. Gonzalez-Zorn, J. Wehland, and J. Kreft. 2001. *Listeria* pathogenesis and molecular virulence determinants. *Clinic. Microbiol. Rev.* 14:584-640.

48. Viteri, G. (Acme Smoked Fish Corp.). 2009. Personal communication.

49. Vogel, B., F. Vivian, O. Bente, G. Lone, and A. Peter. 2004. High-resolution genotyping of *Listeria monocytogenes* by fluorescent amplified fragment length polymorphism analysis compared to pulsed-field gel electrophoresis, random amplified polymorphic DNA analysis, ribotyping, and PCR-restriction fragment length polymorphism analysis. *J. Food Prot.* 67:1656-1665.

50. Vogel, B. F., Y. Y. Ng, G. Hyldig, M. Mohr, and L. Gram. 2006. Potassium lactate combined with sodium diacetate can inhibit growth of *Listeria monocytogenes* in vacuum-packed cold-smoked salmon and has no adverse sensory effects. *J. Food Prot.* 69:2134-2142.

51. Walker, S. J., P. Archer, and J. G. Banks. 1990. Growth of *Listeria monocytogenes* at refrigeration temperatures. *J. Appl. Bacteriol.* 68:157-162.

52. Ward, T. J., L. Gorski, M. K. Borucki, R. E. Mandrell, J. Hutchins, and K. Pupedis. 2004. Intraspecific phylogeny and lineage group identification based on the *prfA* virulence gene cluster of *Listeria monocytogenes. J. Bacteriol.* 186:4994-5002.

52. Wiedmann, M., J. Bruce, C. Keating, E. Johnson, P. L. McDonough, and C. Batt. 1997. Ribotypes and virulence gene polymorphisms suggest three distinct *Listeria*

monocytogenes lineages with differences in pathogenic potential. *Infect. Immun.* 65:2707-2716.

53. Yoon, K. S., C. N. Burnette, K. A. Abou-Zeid, and R. C. Whiting. 2004. Control of growth and survival of *Listeria monocytogenes* on smoked salmon by combined potassium lactate and sodium diacetate and freezing stress during refrigeration and frozen storage. *J. Food Prot.* 67:2465-2471.

CHAPTER 2

DIRECTIONS FOR FUTURE RESEARCH

This research developed a statistical methodology to prove synergistic action of growth inhibitors by decomposing the effects of growth inhibiting treatments into effects on individual growth parameters and then testing for statistically significant interactions between inhibiting treatments. As this work tested only one level of each treatment using only phenotypic measures (growth curves), neither dose dependant variations in synergistic effect, nor the mechanisms of the observed synergy could be elucidated. Therefore, future research could focus on:

Synergy Optimization and Screening

One reason that processors are interested in formulating products with synergistic growth inhibitors is that they suggest a method to achieve greater growth inhibition at less cost (economic, organoleptic, etc.) than with individual inhibitor formulations. While the data presented in this thesis prove the synergy is real, they do not answer the industrial relevant questions of which combination levels provide the maximum synergy benefit, or which combination of levels would provide effective growth inhibition at the least cost. These questions could be addressed by using response surface models, e.g. [11], to test multiple levels of inhibitors and determine (simplest) where the combination where maximum synergy occurs or (more relevant) the inhibitor combination function that maximizes synergy for all levels of overall growth inhibition. As response surface methods require the testing of many different treatment levels, the result from this work that that lag phase and maximum growth rate are highly correlated justify using single endpoint growth measures, such as time-to-detection [9], that are cheaply measured using automated plate readers to lower the time and cost of designed experiments. Such high-throughput growth methods can also be used to screen novel growth inhibitors, as in [6], although experiments should

be performed at industrially relevant cold temperatures and with Analysis of Variance, as opposed to other classification methods, to prove synergies between combinations.

Mechanisms of Synergistic Growth Inhibition

The classic theory of how organic acids inhibit growth of bacteria and yeasts - the undissociated acid in solution diffuses across the cell membrane, dissociates into non-diffusible hydrogen cations and acid anions, and accumulation of intracellular ions is thought to have many growth inhibiting effects including reduction of the proton motive force, reduction of intracellular pH, interference with microbial metabolism, and anion toxicity [4, 5, 8] – cannot explain why some organic acids, such as lactate and diacetate, show synergistic effects, as opposed to additive effects stemming from similar mechanisms of action. While a few studies [1-3, 7] have begun to define the transcriptomic bacterial response to single organic acid stresses, or the proteomic response to the combination of two organic acids [10], these studies are all designed to determine the response to single treatments, not combinations. To accurately study the mechanism of *synergy* between two inhibitors, studies should be designed to gather transcriptomic or proteomic data analogous to this phenotypic data, e.g. microarray hybridization between control, lactate, diacetate, and combination treatments, and tested for statistically significant interactions in gene or protein expression. Mapping these results to physiological pathways may indicate where these organic acids have emergent synergistic properties and suggest targets for the rational design of novel growth inhibiting combination treatments.

REFERENCES

1. Arnold, C.N., J. McElhanon, A. Lee, R. Leonhart, and D.A. Siegele. 2001. Global Analysis of *Escherichia coli* Gene Expression during the Acetate-Induced Acid Tolerance Response. *J Bacteriol*. 183(7):2178-2186.
2. Beek, A.T., B.J.F. Keijser, A. Boorsma, A. Zakrzewska, R. Orij, G.J. Smits, and S. Brul. 2008. Transcriptome Analysis of Sorbic Acid-Stressed *Bacillus subtilis* Reveals a Nutrient Limitation Response and Indicates Plasma Membrane Remodeling. *J Bacteriol*. 190(5):1751-1761.
3. Bowman, J.P., K.J. Lee Chang, T. Pinfold, and T. Ross. 2010. Transcriptomic and phenotypic responses of *Listeria monocytogenes* strains possessing different growth efficiencies under acidic conditions. *Appl Environ Microbiol*. 76(14):4836-4850.
4. Brul, S. and P. Coote. 1999. Preservative agents in foods: Mode of action and microbial resistance mechanisms. *Int J Food Microbiol*. 50(1-2):1-17.
5. Doores, S. 2005. Organic Acids. p. 91-142. *In* P.M. Davidson, J.N. Sofos, and A.L. Branen(ed.), *Antimicrobials in Food*. CRC press. Boca Raton, FL.
6. Gutierrez, J., C. Barry-Ryan, and P. Bourke. 2008. The antimicrobial efficacy of plant essential oil combinations and interactions with food ingredients. *Int J Food Microbiol*. 124(1):91-97.
7. Kitko, R.D., R.L. Cleeton, E.I. Armentrout, G.E. Lee, K. Noguchi, M.B. Berkmen, B.D. Jones, and J.L. Slonczewski. 2009. Cytoplasmic Acidification and the Benzoate Transcriptome in *Bacillus subtilis. PLoS ONE*. 4(12):e8255.
8. Lado, B. and A. Yousef. 2007. Characteristics of *Listeria monocytogenes* important to food processors. p. 157-213. *In* E. Ryser and E. Marth(ed.), *Listeria, listeriosis, and food safety*. CRC Press.

9. Lambert, R.J.W. and E. Bidlas. 2007. An investigation of the gamma hypothesis: a predictive modelling study of the effect of combined inhibitors (salt, pH and weak acids) on the growth of *Aeromonas hydrophila. Int J Food Microbiol*. 115(1):12-28.
10. Phan-Thanh, L., F. Mahouin, and S. Aligé. 2000. Acid responses of *Listeria monocytogenes. Int J Food Microbiol*. 55(1-3):121-126.
11. Valero, A., F. Pérez-Rodríguez, E. Carrasco, R.M. García-Gimeno, and G. Zurera. 2006. Modeling the Growth Rate of *Listeria Monocytogenes* Using Absorbance Measurements and Calibration Curves. *J Food Sci*. 71(7):M257-M264.

APPENDIX

Table A1. Growth parameters for each strain grown in BHI broth at 7°C in the presence or absence of sodium diacetate and/or potassium lactate.

Treatment[a]	Strain	Lineage	λ [days]		u_{max} [log(CFU/ml)/day]		N_0 [log(CFU/ml])		N_{max} [log(CFU/ml)]	
			rep1	rep2	rep1	rep2	rep1	rep2	rep1	rep2
CTRL	c1-122	1	2.8	3.3	0.58	0.53	3.4	3.5	8.7	8.5
CTRL	f2-693	1	2.7	2.2	0.47	0.58	3.4	3.5	8.3	8.7
CTRL	f6-366	1	2.2	2.1	0.56	0.60	3.4	3.5	8.8	8.9
CTRL	j1-175	1	2.7	2.4	0.49	0.60	3.5	3.4	8.4	8.7
CTRL	j2-064	1	2.6	2.5	0.51	0.51	3.4	3.4	8.6	8.5
CTRL	r2-154	1	3.2	2.2	0.47	0.58	3.3	3.2	8.2	8.4
CTRL	r2-182	1	2.4	2.4	0.53	0.49	3.2	3.4	8.7	8.4
CTRL	f2-032	2	2.7	2.4	0.50	0.48	3.3	3.2	8.6	8.3
CTRL	f2-216	2	3.1	3.4	0.58	0.52	3.4	3.4	8.6	8.4
CTRL	f2-237	2	2.3	3.0	0.49	0.50	3.2	3.6	8.6	8.6
CTRL	f2-515	2	3.0	2.1	0.47	0.60	3.3	3.4	8.7	8.8
CTRL	j2-003	2	3.1	2.2	0.51	0.62	3.3	3.3	8.5	8.9
CTRL	r2-559	2	2.5	2.3	0.58	0.69	3.4	3.4	8.9	9.0
SDA	c1-122	1	2.8	2.6	0.55	0.54	3.5	3.5	8.8	8.7
SDA	f2-693	1	2.4	2.3	0.53	0.46	3.4	3.6	8.7	8.6
SDA	f6-366	1	3.5	2.8	0.48	0.49	3.5	3.5	8.6	8.8
SDA	j1-175	1	2.3	2.6	0.53	0.53	3.2	3.5	8.9	8.6
SDA	j2-064	1	2.4	2.3	0.53	0.47	3.5	3.3	8.8	8.4
SDA	r2-154	1	2.0	2.9	0.50	0.52	3.2	3.2	8.5	8.6
SDA	r2-182	1	2.3	3.0	0.55	0.51	3.2	3.3	8.9	8.6
SDA	f2-032	2	2.8	3.0	0.55	0.53	3.3	3.5	8.9	8.5
SDA	f2-216	2	2.8	3.1	0.55	0.51	3.4	3.0	8.9	8.7
SDA	f2-237	2	2.5	2.5	0.55	0.52	3.3	3.4	8.9	8.8
SDA	f2-515	2	2.5	2.7	0.54	0.49	3.4	3.4	9.0	8.8
SDA	j2-003	2	2.7	3.1	0.54	0.52	3.4	3.6	9.0	8.8
SDA	r2-559	2	3.2	4.0	0.49	0.51	3.4	3.5	8.9	8.3
PL	c1-122	1	2.6	3.2	0.54	0.53	3.5	3.5	8.7	8.5
PL	f2-693	1	2.3	3.1	0.46	0.57	3.6	3.5	8.6	8.5
PL	f6-366	1	2.8	3.7	0.49	0.54	3.5	3.5	8.8	8.4
PL	j1-175	1	2.6	4.0	0.53	0.54	3.5	3.4	8.6	8.2
PL	j2-064	1	2.3	3.5	0.47	0.50	3.3	3.3	8.4	8.2
PL	r2-154	1	2.9	3.5	0.52	0.47	3.2	3.5	8.6	8.1
PL	r2-182	1	3.0	3.8	0.51	0.44	3.3	3.3	8.6	7.9
PL	f2-032	2	3.0	4.6	0.53	0.51	3.5	3.5	8.5	8.0

Table A1. (Continued)

PL	f2-216	2	3.1	4.2	0.51	0.48	3.0	3.4	8.7	8.5
PL	f2-237	2	2.5	3.3	0.52	0.51	3.4	3.4	8.8	8.4
PL	f2-515	2	2.7	2.7	0.49	0.50	3.4	3.4	8.8	8.6
PL	j2-003	2	3.1	3.3	0.52	0.57	3.6	3.5	8.8	8.6
PL	r2-559	2	2.8	6.3	0.52	0.36	3.5	3.5	9.0	8.2
PLSDA	c1-122	1	6.3	6.4	0.36	0.30	3.5	3.5	8.2	8.2
PLSDA	f2-693	1	7.6	4.5	0.29	0.38	3.4	3.4	7.9	8.3
PLSDA	f6-366	1	5.0	5.8	0.37	0.37	3.5	3.5	8.6	8.3
PLSDA	j1-175	1	7.9	4.8	0.33	0.40	3.4	3.4	8.1	8.3
PLSDA	j2-064	1	6.7	6.1	0.35	0.30	3.6	3.4	8.2	8.1
PLSDA	r2-154	1	9.3	4.5	0.31	0.33	3.3	3.2	8.0	8.3
PLSDA	r2-182	1	6.5	6.7	0.34	0.29	3.3	3.4	8.1	8.2
PLSDA	f2-032	2	7.4	10.2	0.26	0.27	3.3	3.4	7.9	7.5
PLSDA	f2-216	2	7.5	7.8	0.39	0.31	3.6	3.4	8.2	8.1
PLSDA	f2-237	2	7.3	8.4	0.30	0.31	3.5	3.4	8.4	8.2
PLSDA	f2-515	2	8.3	4.0	0.29	0.43	3.4	3.3	8.2	8.4
PLSDA	j2-003	2	7.2	5.2	0.27	0.39	3.2	3.3	8.2	8.5
PLSDA	r2-559	2	5.4	5.5	0.39	0.39	3.5	3.5	8.6	8.4

[a]CTRL, control; SDA, sodium diacetate; PL, potassium lactate; PLSDA, combination of sodium diacetate and potassium lactate.

Table A2. Correlation coefficients between regression parameters for the entire data set (overall correlation) and for the parameters grouped by growth inhibitor treatment (CTRL, SDA, PL, and PLSDA correlations).

Parameter	**λ[a]**	**μ_{max}[a]**	**N_0[a]**	**N_{max}[a]**
Overall correlation, n=104				
λ	1.00	*-0.879*	0.128	*-0.718*
μ_{max}	*-0.88*	1.000	-0.023	*0.705*
N_0	0.13	-0.023	1.000	-0.015
N_{max}	*-0.72*	*0.705*	-0.015	1.000
CTRL treatment correlation, n=26				
λ	1.000	*-0.477*	0.234	*-0.534*
μ_{max}	*-0.477*	1.000	0.144	*0.781*
N_0	0.234	0.144	1.000	0.126
N_{max}	*-0.534*	*0.781*	0.126	1.000
SDA treatment correlation, n=26				
λ	1.000	-0.111	0.157	0.009
μ_{max}	-0.111	1.000	-0.127	*0.560*
N_0	0.157	-0.127	1.000	0.031
N_{max}	0.009	*0.560*	0.031	1.000
PL treatment correlation, n=26				
λ	1.000	-0.386	0.051	*-0.556*
μ_{max}	-0.386	1.000	0.387	0.302
N_0	0.051	0.387	1.000	0.182
N_{max}	*-0.556*	0.302	0.182	1.000
PLSDA specific correlation, n=26				
λ	1.000	*-0.732*	-0.024	*-0.714*
μ_{max}	-0.732	*1.000*	0.340	*0.650*
N_0	-0.024	*0.340*	1.000	*0.201*
N_{max}	-0.714	*0.650*	0.201	*1.000*

[a] significant effects ($p<0.05$) are italicized

Table A3. Strains that were discovered to grow slower than average in DM2 during the course of this project. Growth data from the experiments supporting these conclusions follow in Figures A1 to A5. Lineage, serotype, and ribotype data come from pathogen tracker.

FSL designation	Lineage	Serotype	Ribotype	Source	Supporting Figure A#
Strains that failed to reach OD_{480} > 1.0 in DM2					
FSL N3-013	LI	4b	1042B	pate outbreak	3
FSL R2-585	LI	4b	1042B	food epidemic	4
FSL N1-017	LI	4b	1042C	trout in brine	1
FSL R2-435	LI		1042C	smoked seafood	2
FSL J1-167	LI	1/2b	1042B	human, sporadic	2
Strain that grew slowly in DM2 but did eventually reach OD_{480} > 1.0					
FSL J1-194	LI	1/2b[a]	1042B	human, sporadic	1,3,5
FSL R2-503	LI	1/2b	1051B	gastroenteritis outbreak, 1994	3,5
FSL R2-597	LI	1/2b	1051B	epidemic RTE Food (Dairy)	4,5
FSL J1-116	LI	4b	1042B	human epidemic	4,5
FSL J1-126	LI	4b	1038B	human epidemic	4,5
FSL R2-502	LI	1/2b	1051B	food, chocolate milk	4,5
FSL R2-598	LI	1/2b	1051B	human epidemic	4,5

[a] Serogroup designation from multiplex PCR.

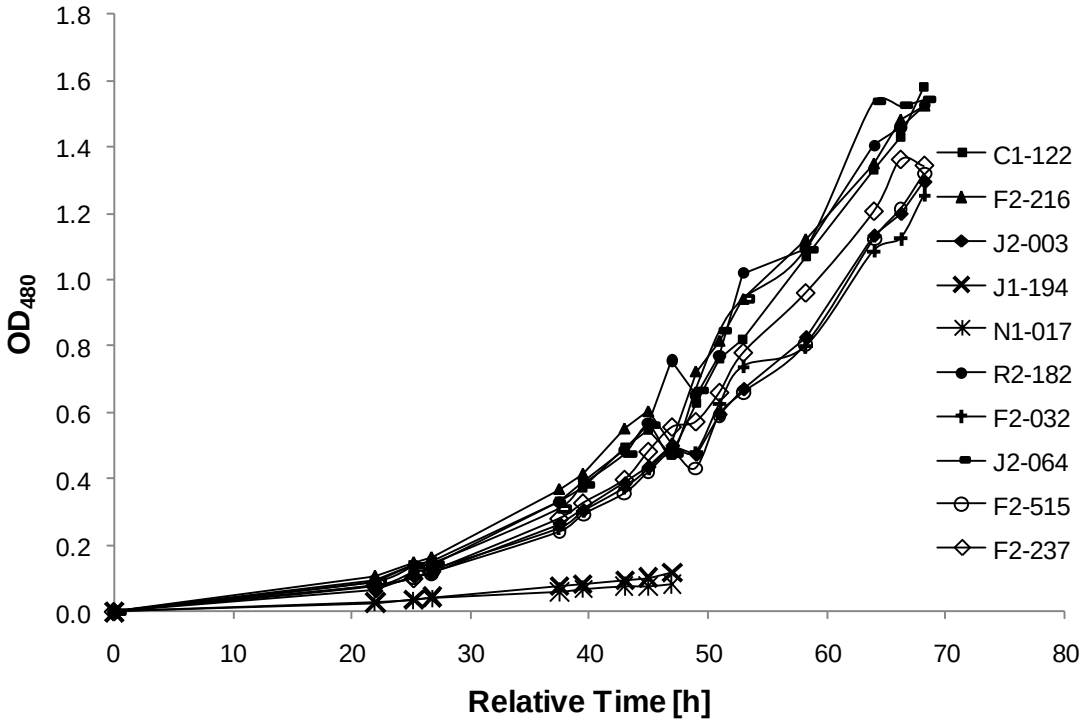

Figure A1. Growth of 13 strains in DM2 on 3-3-2008 in preparation for the paper in this thesis.

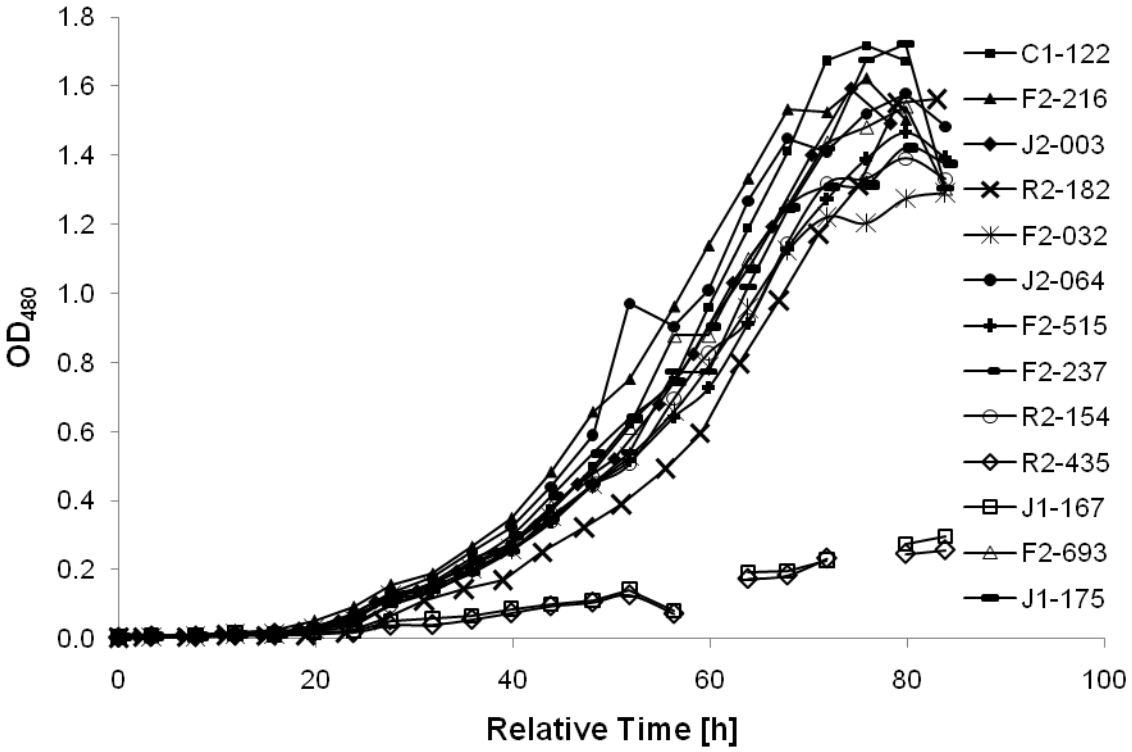

Figure A2. Growth of a revised set of 13 in DM2 on 3-10-2008 in preparation for the paper in this thesis.

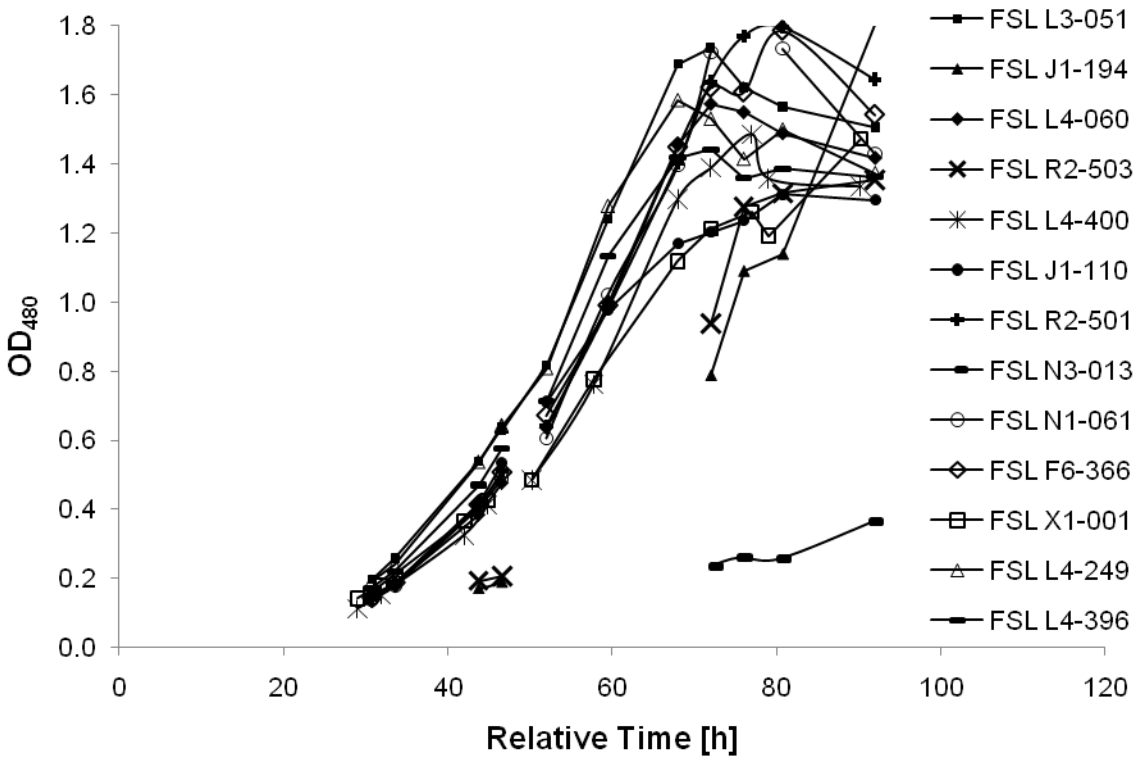

Figure A3. Growth of 20 strains (not all shown) in DM2 on 7-12-09 in preparation for work on a New York Sea Grant project.

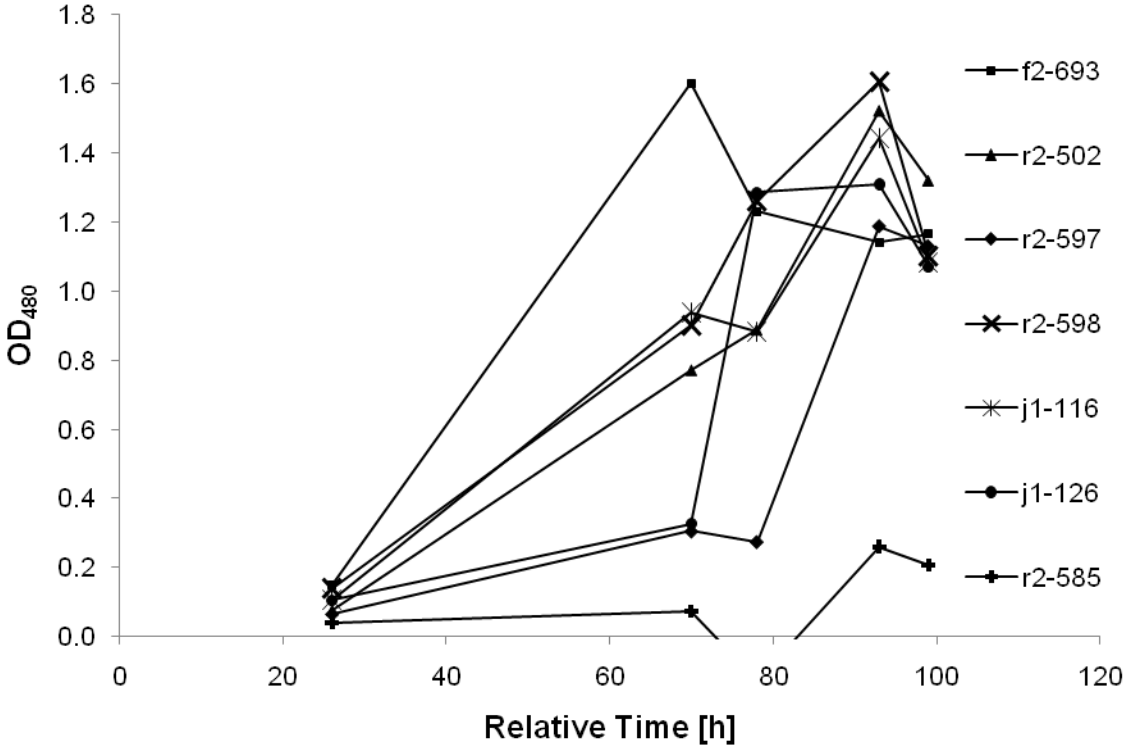

Figure A4. Growth of 7 strains in DM2 on 7-16-2009 to in preparation for a work on a New York Sea Grant project.

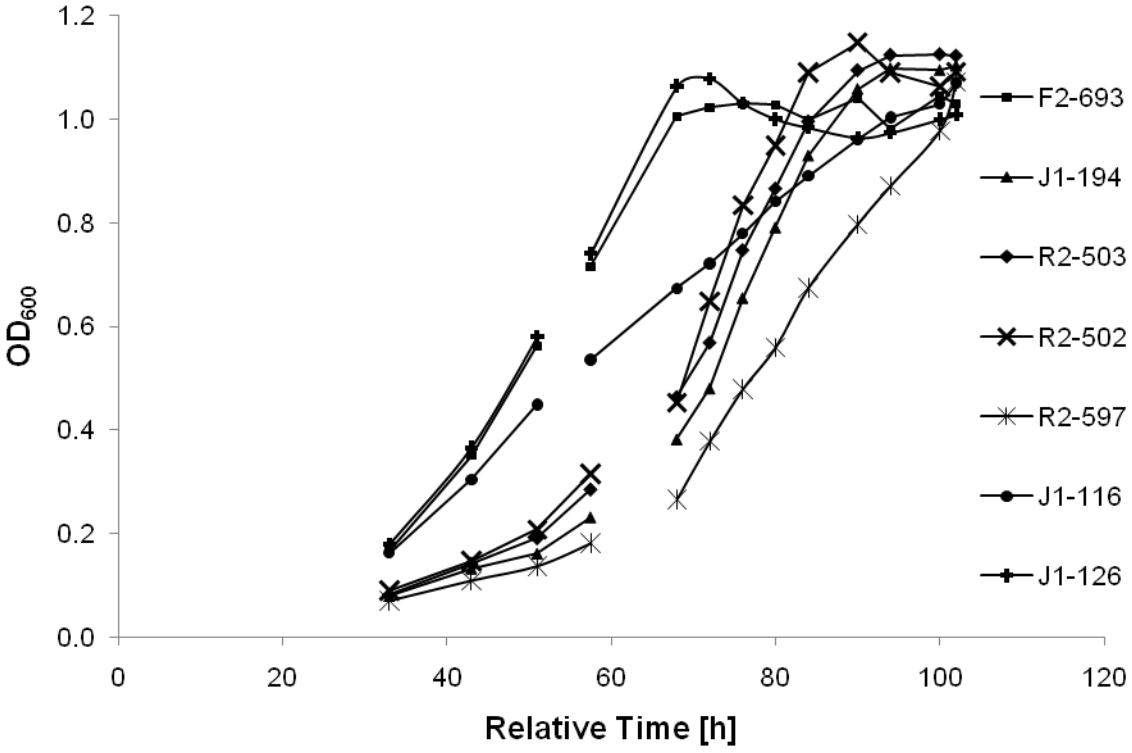

Figure A5. Re-test of the growth of the strains in Figure A4 in DM2 on 9-14-2009.