Journal of Food Protection, Vol. 84, No. 5, 2021, Pages 802–810
https://doi.org/10.4315/JFP-20-435
Published 2021 by the International Association for Food Protection

Research Paper

Enabling Cost-Effective Screening for Antimicrobials against *Listeria monocytogenes* in Ham

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MS 20-435: Received 28 October 2020/Accepted 8 December 2020/Published Online 10 December 2020

ABSTRACT

Ready-to-eat meat products, such as deli ham, can support the growth of *Listeria monocytogenes* (LM), which can cause severe illness in immunocompromised individuals. The objectives of this study were to validate a miniature ham model (MHM) against the ham slice method and to screen antimicrobial combinations to control LM on ham by using response surface methology (RSM) as a time- and cost-effective high-throughput screening tool. The effect of nisin (Ni), potassium lactate and sodium diacetate, lauric arginate (LAG), lytic bacteriophage (P100), and ϵ-polylysine (EPL) added alone, or in combination, were determined on the MHM over 12 days of storage. Results showed the MHM accurately mimics the ham slice method because no statistical differences were found ($P = 0.526$) in the change of LM cell counts in MHM and slice counts after 12 days of storage at 4°C for treated and untreated hams. The MHM was then used to screen antimicrobial combinations by using an on-face design and three center points in a central composite design. The RSM was tested by using a cocktail of five LM strains isolated from foodborne disease outbreaks. Three levels of the previously mentioned antimicrobials were used in combination for a total of 28 runs performed in triplicate. The change of LM cell counts were determined after 12 days of storage at 4°C. All tested antimicrobials were effective on reducing LM cell counts on ham when added alone. A significant antagonistic interaction ($P = 0.002$) was identified by the RSM between LAG and P100, where this antimicrobial combination caused a 2.2-log CFU/g change of LM cell counts after 12 days of storage. Two interactions, between Ni and EPL ($P = 0.058$), and Ni and P100 ($P = 0.068$), showed possible synergistic effects against LM on the MHM. Other interactions were clearly nonsignificant, suggesting additive effects. In future work, the developed MHM in combination with RSM can be used as a high-throughput method to analyze novel antimicrobial treatments against LM.

HIGHLIGHTS

- A miniature ham model was validated against ham slices.
- Response surface methodology was used to analyze antimicrobial combinations on ham.
- A time- and cost-effective alternative to control LM on ham was developed.

Key words: Antimicrobials; Ham; *Listeria monocytogenes*; Response surface methodology

Listeria monocytogenes (LM) is a foodborne pathogen responsible for causing listeriosis upon consumption. LM is a threat to the safety of consumers of ready-to-eat (RTE) meat products because of the ability of the bacterium to grow at temperatures ranging from 0 to 45°C, to resist high salt concentrations, and to thrive under a wide range of pH values *(7)*. RTE meat products are easily contaminated after processing (e.g., slicing or chopping) *(23)*. These products have a long shelf life by storage at 4 to 8°C and are usually eaten without further processing, LM can grow and proliferate to dangerous levels in RTE meat products during storage *(36)*. A 2003 risk assessment showed that deli meats, such as ham, showed the greatest risk of listeriosis of 23 assessed food categories *(10)*. Even if relative risks have changed in recent years (e.g., increased LM from RTE produce), listeriosis cases linked to deli meats are still significant in the United States and worldwide *(4)*.

Adding antimicrobial agents to RTE meat products is a common practice in the meat industry to control LM contamination throughout storage *(15)*. Generally recognized as safe (GRAS) antimicrobials, such as nisin (Ni), potassium lactate–sodium diacetate (PLSDA), lauric arginate (LAG), bacteriophages (P100), and ϵ-polylysine (EPL), are commercially available to use on RTE meat products. Previous studies have shown that the antimicrobial concentrations needed to control LM are different when tested on broth systems compared with testing directly in the food matrices *(26)*. Because food matrices are compositionally more complex, compared with laboratory media systems, often increased doses of antimicrobials are required to control bacterial growth *(8)*. For example, 200

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ppm of LAG (maximum limit permitted on food) extended the lag phase of LM in brain heart infusion (BHI). However, no effect on growth parameters was shown when 200 ppm of LAG was applied to smoked salmon *(16),* perhaps because of the high protein content in the cold-smoked salmon. Other studies have shown that LAG bacteriostatic effect is affected by high protein concentrations *(25)*. Furthermore, the bactericidal activity of Ni was measured in vitro and on RTE bologna sausage *(8)*. The MIC of Ni (25 ppm) was applied directly on the sausage. Results showed the tested Ni concentration did not reduce LM counts on sausage, compared with the control after 7 days of storage. These studies highlight the need for testing the antimicrobials in the food matrix of interest.

However, screening methods to study the effect of antimicrobials to control the growth of LM on RTE meat products are laborious and expensive, because they typically require the use of whole food products, such as slices of deli ham *(6, 11, 17)*. Antimicrobials and pathogen cultures are applied to the surface of the slice of ham. Then, the slice of ham is diluted in buffer and enumerated with selective media. Laboratory food models are a cost-effective alternative to monitor antimicrobial effectiveness. Laboratory food models have been developed for assessing antilisterial antimicrobials, such as for Queso Fresco *(32)*. Even though laboratory ham models have been proposed before *(2, 33, 34),* none of these models have been validated against the ham slice method. A model is needed that accurately mimics the ham slice method, while increasing the efficiency of antimicrobial screening, to develop cost-effective antilisterial treatments on ham.

Response surface methodology (RSM) is a tool for process optimization. It is a multivariate statistical technique that depicts the behavior of a data set with the purpose of making statistical predictions *(3)*. RSM models predict the effect of independent variables and their interactions on a dependent variable by using statistically efficient experimental designs, such as central composite, to strategically test effects of independent variables. This allows for fewer combinations, and therefore fewer individual tests, than a full factorial design. Although RSM has been implemented to optimize ham formulation, such as sodium, pH, moisture, and limited antimicrobials *(9, 22),* it has not been used to screen many antimicrobials at different levels directly on ham.

The present study aims to validate a miniature ham model (MHM) against the typical whole slice ham method. Also, we propose the implementation of RSM and the MHM as a time- and cost-effective high-throughput screening tool. Therefore, we tested several GRAS antimicrobials (Ni, PLSDA, LAG, P100, and EPL) and combinations against LM cell counts during storage on deli ham.

MATERIALS AND METHODS

Bacterial strains and inoculum preparations. The LM strains used in this study were all recovered from cases related to foodborne disease (Table 1), which suggests the ability of these strains to endure RTE meat stress. The LM strains were combined as a cocktail to create a conservative estimate of the effect antimicrobials can have on bacterial proliferation because whichever strain is most resistant to the specific antimicrobial treatment will likely predominate after storage. The LM cocktail was prepared as described before *(14)*. Briefly, the LM cocktail strains were inoculated in BHI (Difco, BD, Sparks, MD) from glycerol stocks stored at −80°C. The bacteria were grown overnight in 10 mL of BHI broth for 18 h at 37°C with an agitation of 250 rpm. For stress response adaption of LM in ham, the cultures were then passed to 5 mL of BHI media to grow for 24 h at room temperature (∼22°C) with no shaking, as described before *(19)*. The LM cocktail was prepared by adding 1 mL of each culture to a sterile tube. Before inoculation, the LM cocktail was diluted in phosphate-buffered saline (PBS) solution (KCl, 200 mg/L; KH_2PO_4, 200 mg/L; NaCl, 8 g/L; Na_2HPO_4, 1.15 g/L, and pH 7.2; MP Biomedicals, LLC, Solon, OH) to achieve a 4-log CFU inoculum. After the incubation period, bacterial enumeration was carried out to confirm the strains were being added to the cocktail in a 1:1 ratio (Supplemental Table S1).

TABLE 1. *Foodborne disease outbreak–associated LM strains used in the cocktail for this study*

L. monocytogenes strain	Serotype	Source of isolation
NRRL B-33419	1/2a	Human, epidemic, sliced turkey
NRRL B-33424	1/2b	Human, epidemic, chocolate milk
NRRL B-33420	4b	Food, epidemic, RTE meat products
NRRL B-33513	4b	Food, epidemic, pate
NRRL B-33104	4b	Food, epidemic, cheese

Sample preparation. Ham was made at the University of Illinois Meat Science Laboratory, a commercial sales facility regulated by the U.S. Department of Agriculture's Food Safety and Inspection Service. The ham formula was based on the retail ham sold by the Meat Science Laboratory. Briefly, sodium hexametaphosphate (0.5% by weight) was added to a mixture of ice and water in the same proportion (18% by weight) until fully dissolved. Then, NaCl (2.2% by weight), celery powder (0.51% by weight; Excalibur Seasoning Company, Pekin, IL), and cherry powder (0.43% by weight; Excalibur Seasoning Company) were mixed into the brine. Dextrose (2.46% by weight) was added last to the brine. Muscle-derived pork leg cuts (75.9% by weight) were diced into cubes (∼2.5 cm^3) and vacuum tumbled with the brine for 2 h. The mixture was stuffed into nylon casings and smoked at 76°C heat for 7 h. After chilling, the ham was sliced into 2.5-mm slices, individually vacuum sealed, and frozen. The ham had a protein fat free of 21.8 g/kg of ham, which determines the type of standard identity of the product, with this product being labeled as "ham." This product had a final sodium content of 1.3% and a water activity of 0.963 ± 0.001 at 4°C.

Antimicrobials. Five commercially available antimicrobials with GRAS status *(31)* were acquired for use in this study, which are listed in Table 2. Nisaplin (Dansico, Beaminster, UK) contains the bacteriocin Ni (2.5%, w/w) and was diluted in sterile distilled water until a final concentration of 25 ppm. LAG (10% LAG; CytoGuardLA 20 Antimicrobial, A&B Ingredients, Fairfield, NJ) was diluted in sterile distilled water for a final concentration of 200 ppm. The organic acid combination, L-potassium lactate (54.5 to 57.5%, w/w) and sodium diacetate (3.7 to 4.3%, w/w; Opti.Form PD 4, Corbion Purac, Amsterdam, The Netherlands) was used at an upper limit of 3.5% (company recommendation for sliced ham). PhageGuard Listex P100 (Micreos Food Safety,

TABLE 2. *Antimicrobials used in the MHM response surface methodology*

Antimic	Lower usage level	Upper usage level
Ni	0 ppm	25 ppm
P100	0 PFU/g	2×10^7 PFU/g
Potassium lactate + sodium diacetate	0% (w/w)	3.5% (w/w)
LAG	0 ppm	200 ppm
EPL	0 ppm	500 ppm

Wageningen, The Netherlands) was used at 2×10^7 PFU/g (company recommendation for sliced ham) with a stored concentration of approximately 10^{11} PFU/mL in buffered saline and was maintained at 5°C. EPL (Wilshire Technologies, Princeton, NJ; EPL > 98%, w/w) was diluted in sterile distilled water to a final concentration of 500 ppm. Every solution was prepared freshly on the day of the experiment.

MHM. Individually vacuum-sealed 2.5-mm ham slices were defrosted at ~5°C for at least 12 h before the experiment. A flame-sterilized circular metal cutter (7.9-mm diameter) was used to punch uniform ham disks into 2-mL microcentrifuge tubes; each ham weighed 149 ± 5 mg. Then, 50 μL of antimicrobials were inoculated onto the ham. The tubes were centrifuged (Sorvall Legend Micro 17, Thermo Fisher Scientific, Waltham, MA) at 6,200 × *g* for 2 min. Hams were inoculated with 50 μL of LM cocktail and centrifuged at 6,200 × *g* for 3 min. Excess liquid was removed from the tubes. Each replicate had an initial average inoculum level of 4.25 ± 0.15 log CFU/g. The experiment was performed in technical replicates and biological triplicates.

Ham slice method. Individually vacuum-sealed 2.5-mm ham slices were defrosted at ~5°C for at least 12 h before the experiment. Slices were transferred to sterile Whirl-Pak Plain Blender Bags (55 oz [1,627 mL]; Uline Co., Pleasant Prairie, WI), where antimicrobials diluted in sterile distilled water were aseptically spread onto both sides of the ham slice and let dry for 30 min at 5°C. The ham was then inoculated with 1.5 mL of LM cocktail and let sit for 30 min at 5°C for cell attachment. Excess liquid was removed (over half liquid recovered), the Whirl-Pak bag was sealed, and the hams were stored at 4°C. The LM cocktail strains were used for the comparison of the MHM to the ham slice method, which was tested in triplicate, with three replicates for the experiment.

Determination of growth at end point. A 12-day end point for the MHM hams and ham slice method was determined by adhering to the federal regulations for delicatessen ham *(29, 30)*. Taking the maximum amount of time for each recommendation, 12 days was used for the time course of ham slices after being thawed and opened.

After 12 days, the MHM was suspended 1:10 of PBS. The MHM was disrupted by vortexing (Vortex-Genie 2, VWR, Rochester, NY) for 5 min to remove the cells from the ham surface. Disruption by vortexing, stomaching, and bead beating was compared by using an unbiased all pair comparison (Tukey-Kramer test). Cell recovery by vortexing was not statistically different from using a stomacher ($P = 0.241$). Disruption by bead beating was statistically different from using a stomacher ($P = 0.038$); however, bead beating and vortexing were not statistically different ($P = 0.163$; Table S2).

The ham slices were suspended 1:5 in PBS and hand massaged for 5 min. Additional serial dilutions in PBS were made to the ham solutions. Appropriate dilutions were enumerated on polymyxin–acriflavin–lithium chloride–ceftazidime–esculin–mannitol (PALCAM) *Listeria*-selective agar (EMD Millipore, Darmstadt, Germany) with the *Listeria* selective supplement (EMD Millipore) in duplicate with a spiral plater (Eddy Jet Spiral Plater, Neutec Group Inc., Farmingdale, NY). Plates were incubated at 37°C for 48 h before counting with a colony counter (IUL Flash and Go, Neutec Group Inc.).

Treatments that showed no growth on the PALCAM plates after incubation were further analyzed by enrichment. The miniature hams' original 1:10 dilution on PBS (100 μL) was transferred to 5 mL of BHI broth (Difco, BD) and incubated for 24 h at 37°C. Enrichment tubes were considered positive if turbidity was seen on the tube after incubation. The limit of detection of the enrichment is considered to be 1 CFU/mL.

Experimental design and data analysis. To validate the MHM, bacterial counts after 12 days were analyzed by using JMP Pro 14 statistical software (SAS Institute Inc., Cary, NC). A two-way analysis of variance (ANOVA) was performed to determine statistical differences ($P < 0.05$) between the MHM and ham slice method, as well as statistical difference between treated and untreated hams.

JMP Pro 14 was used for the design of the experiment. The RSM is an on-face design (axial points do not exceed limits) with five independent variables (Ni, LAG, PLSDA, P100, and EPL). The dependent variable (Y factor) was *Listeria* spp. cell counts after 12 days (log CFU per gram; $N_i - N_0$) on the MHM. There are three center points in the central composite design, shown in Table 3. The central composite design had 28 total treatments in the MHM, done in triplicate. A multiple regression analysis was used to determine a prediction equation for the reduction of LM (log CFU per gram; $N_i - N_0$). The regression model included all independent variables (Ni, LAG, PLSDA, P100, and EPL), with the interaction and quadratic terms. For purposes of interpretation, any statistically significant interaction terms would be considered evidence for either synergism (if the sign is negative) or antagonism (if the sign is positive). JMP Pro 14 was then used to analyze the collected outcome data by using the standard RSM package. For this analysis, all treatments with cell counts less than the limit of detection (i.e., < 2.25 log CFU/g) were analyzed at the value of 0.82 log CFU/g (limit of detection of preenrichment).

RESULTS

Validation of an MHM. To validate the MHM, the model was compared with the ham slice method with (50 ppm of Ni) and without (control) added antimicrobials for similar cell counts after a 12-day incubation (Fig. 1). This experiment was performed in biological quadruplicates. The 12-day end point was determined by taking the maximum amount of time deli meats should be stored for. On the basis of the "Food Code 2013" *(30),* RTE food products, such as deli meats, should be held in a food establishment stored at 4°C or less for a maximum of 7 days once the package has been opened. In addition, the "Refrigeration and Food Safety" guideline *(29)* recommends that a consumer should

TABLE 3. *Central composite design for the MHM RSM*[a]

Ham	Pattern[b]	Ni (ppm)	PLSDA (%)	LAG (ppm)	P100 (PFU/g)	EPL (ppm)	Δ *L. monocytogenes* cocktail		
							Actual counts (log CFU/g)	Predicted counts (log CFU/g)	Residuals (log CFU/g)
1	−−+−+−	0	0	200	2×10^7	0	2.20	2.24	−0.04
2	0a000	12.5	0	100	1×10^7	250	0.75	0.42	0.33
3	A0000	25	1.75	100	1×10^7	250	−1.09	−1.06	−0.03
4	−++−−	0	3.5	200	0	0	−0.46	−0.47	0.01
5	000A0	12.5	1.75	100	2×10^7	250	−0.69	−0.44	−0.25
6	−−−++	0	0	0	2×10^7	500	0.67	0.66	0.01
7	++−−−	25	3.5	0	0	0	−1.52	−1.51	−0.01
8	+−−+−	25	0	0	2×10^7	0	0.01	0.06	−0.05
9	000a0	12.5	1.75	100	0	250	0.05	−0.10	0.15
10	+−+−−	25	0	200	0	0	−0.05	0.04	−0.09
11	++++−	25	3.5	200	2×10^7	0	−1.68	−1.73	0.05
12	−++++	0	3.5	200	2×10^7	500	−1.00	−1.11	0.11
13	−+−−+	0	3.5	0	0	500	−0.37	−0.42	0.05
14	00a00	12.5	1.75	0	1×10^7	250	−0.47	−0.46	−0.01
15	0000a	12.5	1.75	100	1×10^7	0	0.22	0.05	0.17
16	a0000	0	1.75	100	1×10^7	250	0.43	0.49	−0.06
17	−−+−+	0	0	200	0	500	1.30	1.33	−0.03
18	−−−−−	0	0	0	0	0	3.32	3.42	−0.10
19	+−+++	25	0	200	2×10^7	500	0.51	0.50	0.01
20	00000	12.5	1.75	100	1×10^7	250	−0.55	−0.52	−0.03
21	++−++	25	3.5	0	2×10^7	500	−2.44	−2.54	0.10
22	+−−−+	25	0	0	0	500	0.50	0.55	−0.05
23	00A00	12.5	1.75	200	1×10^7	250	−0.74	−0.65	−0.09
24	0000A	12.5	1.75	100	1×10^7	500	−0.87	−0.60	−0.27
25	0A000	12.5	3.5	100	1×10^7	250	−2.51	−2.08	−0.43
26	−+−+−	0	3.5	0	2×10^7	0	−0.62	−0.67	0.05
27	+++−+	25	3.5	200	0	500	−2.74	−2.80	0.06
28	00000	12.5	1.75	100	1×10^7	250	−0.10	−0.52	0.42

[a] Change in the actual and predicted cell counts of LM cocktail after 12 days ($N_0 - N_i$) of storage at 4°C on the MHM. Correlation factor $R^2 = 0.985$ at $\alpha = 0.05$ for the fitted RSM.

[b] −, the low value; +, the high value; 0, the midrange or center value; a, the low axial value; A, the high axial value listed in the following order: Ni, PLSDA, LAG, P100, and EPL. Due to the on-face design, the axial values and corresponding high or low values are the same.

keep fully cooked deli ham slices for no more than 3 to 5 days in their refrigerator before discarding.

Ni has been successfully used to control LM via ham slice method before *(11)*. Surface applying 50 ppm of Ni on the MHM and the ham slice method resulted in a similar change of LM counts. After 12 days, the LM cocktail on untreated slice ham grew to 7.58 ± 0.68 log CFU/g. The untreated MHM grew to 8.02 ± 0.64 log CFU/g. Figure 1 shows the change of LM counts after 12 days of storage by subtracting the counts on day 0 from the counts on day 12 ($N_0 - N_i$). A two-way ANOVA using JMP Pro 14 concluded no statistical difference in the control ham slice and MHM ($P = 0.525$; Fig. 1). When treated with 50 ppm of Ni, LM on slice ham declined to 2.0 ± 0.20 log CFU/g, and LM on the MHM declined to 2.23 ± 0.40 log CFU/g, with a two-way ANOVA concluding no statistical difference ($P > 0.001$; Fig. 1).

Regression analysis. The MHM was used to evaluate the effect of Ni, PLSDA, LAG, P100, EPL, and combinations on the growth of a cocktail of LM. The MHM was treated with 28 combinations (Table 3) of the previously mentioned antimicrobials, and the change in cell counts after 12 days of storage at 4°C were determined.

The RSM establishes two-way interactions between antimicrobial treatments by regression analysis. The whole model with all independent variables and interaction terms is shown in Table 4. The RSM also predicts the quadratic interaction between terms. However, the quadratic terms were not significant ($P > 0.849$). On the basis of the RSM regression analysis, 98% of the variance of LM cell counts can be explained by the model ($R^2 = 0.98$). Therefore, the model can be used to accurately predict the change of LM cell counts.

The RSM model predicts the effect of different levels of antimicrobials added alone, or in combination, to the LM cell counts by a prediction formula (equation 1). The regression coefficients in the equation are weighted to the antimicrobials usage unit. If no antimicrobials were added to the MHM, the LM cocktail would be predicted to grow 3.81 log CFU/g over 12 days of storage at 4°C (Table 3). The predicted counts (Table3) are generated by the model

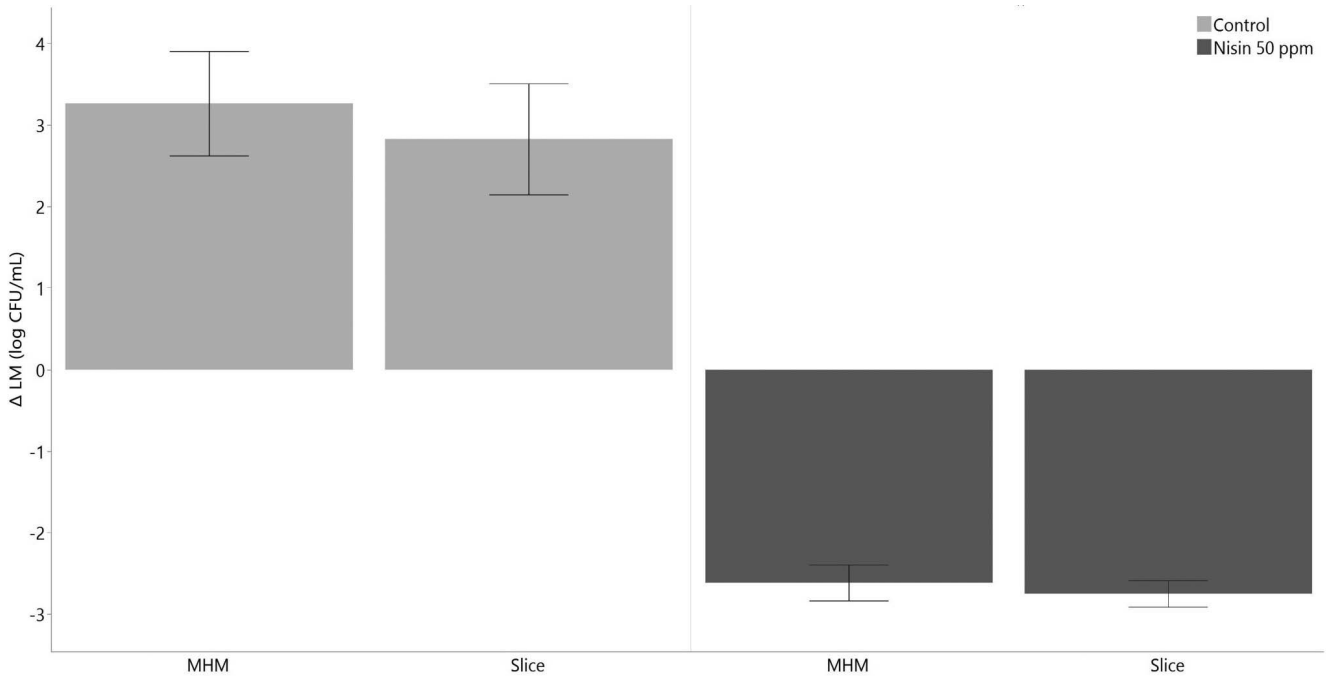

FIGURE 1. *Comparison of the change of LM cocktail cell counts in an MHM and a ham slice model after 12 days ($N_0 - N_i$) at 4°C with a control and Ni treatment. The initial inoculum was 4.8 ± 0.2 log CFU/g. The experiment was performed in biological quadruplicates. A two-way ANOVA showed nonsignificant differences between the MHM and the slice ham ($P < 0.001$), and significant differences were found between treatments (control and Ni 50 ppm; $P = 0.525$).*

using equation 1. The residuals represent the difference between the actual counts and the predicted counts (Table 3). The residual average is -7.1×10^{-4} log CFU/g, suggesting limited bias in prediction. Using the prediction formula is possible to test many different antimicrobial concentrations to achieve a desired bacterial reduction. Optimized, cost-effective, antimicrobial combinations can be achieved by using this formula. The RSM equation is for the predicted change of LM cocktail cell counts after 12 days of storage on the MHM:

$$\begin{aligned}\text{Pred cell counts} = {} & 3.054 - 0.095\text{Ni} - 0.768\text{PLSDA} \\ & - 4.09 \times 10^{-3}\text{LAG} - 8.26 \times 10^{-8}\text{P100} \\ & - 2.42 \times 10^{-4}\text{EPL} \\ & + 1.63 \times 10^{-3}(\text{Ni} - \text{PLSDA}) \\ & + 2.16 \times 10^{-5}(\text{Ni} - \text{LAG}) \\ & - 1.31 \times 10^{-4}(\text{PLSDA} - \text{LAG}) \\ & + 1.38 \times 10^{9}(\text{Ni} - \text{P100}) \\ & + 3.6 \times 10^{-9}(\text{PLSDA} - \text{P100}) \\ & + 5.82 \times 10^{-5}(\text{Ni} - \text{EPL}) \\ & + 3.2 \times 10^{-5}(\text{PLSDA} - \text{EPL}) \\ & + 2.24 \times 10^{-6}(\text{LAG} - \text{EPL}) \\ & + 3.93 \times 10^{-10}(\text{LAG} - \text{P100}) \\ & + 1.08 \times 10^{-11}(\text{P100} - \text{EPL})\end{aligned}$$

where the predicted LM cell count is reported in log CFU/g, Ni, LAG, and EPL in ppm, PLSDA as a percentage of the ham weight, and P100 in PFU/g.

TABLE 4. *Factor significance from RSM regression analysis for predicting change of LM cell counts*[a]

Factor[b]	*P* value
Ni	<0.001
PLSDA	<0.001
EPL	0.004
P100	0.059
LAG	0.242
LAG-P100	0.002
Ni-EPL	0.058
Ni-P100	0.068
PLSDA-P100	0.449
LAG-EPL	0.510
Ni-PLSDA	0.659
Ni-LAG	0.735
P100-EPL	0.747
PLSDA-LAG	0.770
PLSDA-EPL	0.863

[a] This was after 12 days of storage at 4°C on MHM.
[b] Quadratic terms (e.g., Ni-Ni) are not included in the table because all were nonsignificant.

Interpretation. Ni ($P < 0.001$), PLSDA ($P < 0.001$), and EPL ($P = 0.004$) alone were the most effective antimicrobials (Table 4), followed by P100 ($P = 0.059$) and LAG ($P = 0.242$), in the change of LM cell counts on the MHM. The effect of each pair of antimicrobials on the cell counts can be explained by interaction plots (Fig. 2). In general, if the lines are parallel, the two antimicrobials have no interaction and the alteration of one of them does not influence the other. However, when the curves intersect at some point, the two variables have interactive effects. As shown by the model and the interaction plots, there is a significant interaction between LAG and P100 ($P = 0.002$). However, the interaction between these antimicrobials

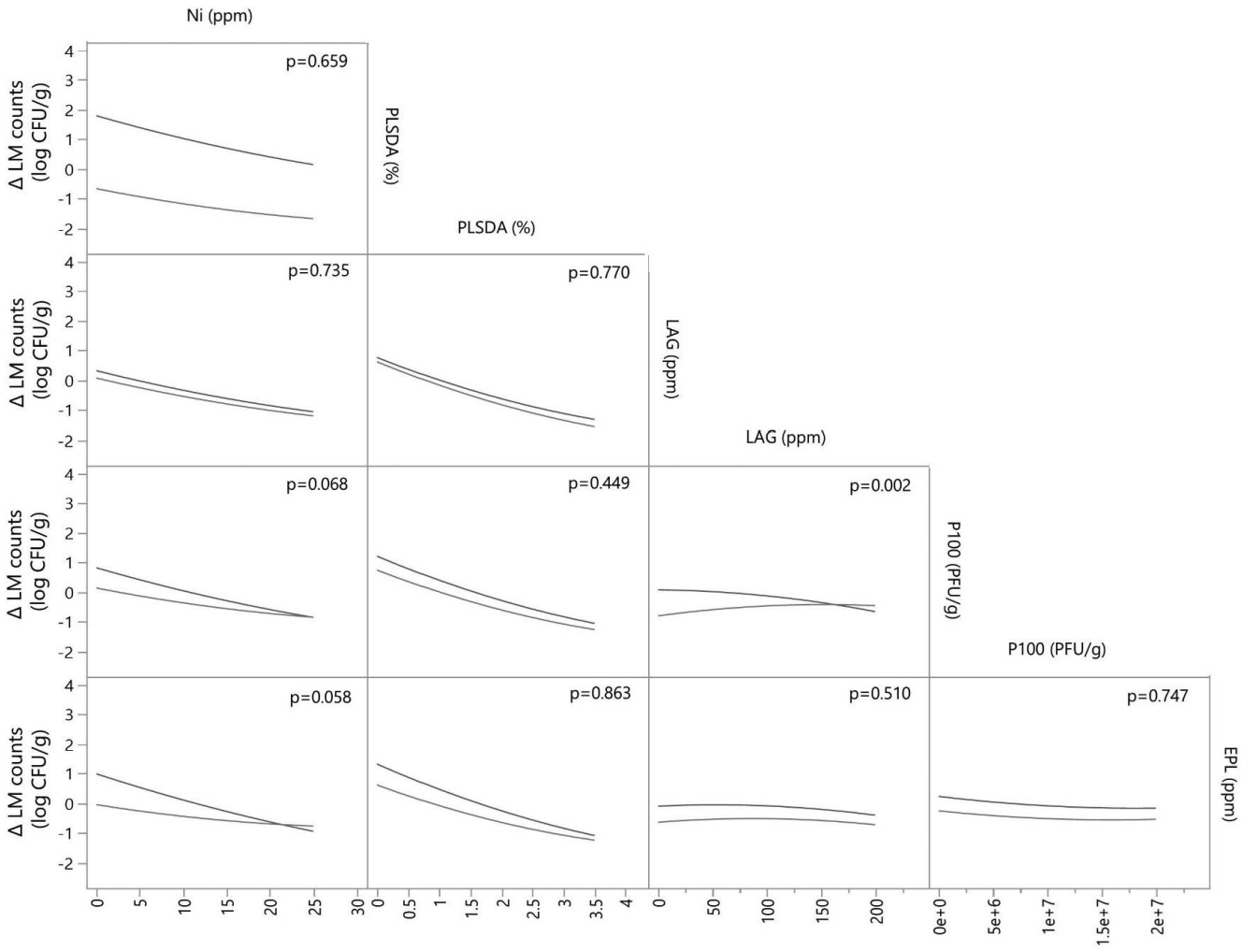

FIGURE 2. *Two-way interaction plot between Ni, PLSDA, LAG, P100, and EPL. Statistically significant interactions were found to be Ni-EPL (*P *= 0.058), Ni-P100 (*P *= 0.068), and LAG-P100 (*P *= 0.002).*

applied in combination to the MHM changed LM cell counts by 2.20 log CFU/g, suggesting a possible antagonism between these antimicrobials.

Surface plots are generated from the RSM prediction equation and show two-way interactions between antimicrobials. The grid design on the surface plots can be used for a sense of the predicted cell counts of any given combination between two antimicrobials within the experimentally tested concentration range. The labels on the grid represent the predicted change in cell counts of LM on MHM after 12 days of storage at 4°C. Positive numbers represent higher cell counts on day 12 than those on day 0, and negative numbers mean the cell counts on day 12 are less than those on day 0. The combination of Ni-PLSDA ($P = 0.659$) did not show any interaction (Fig. 2). However, the addition of these two antimicrobials to the MHM has a linear effect on the LM cell counts (Fig. 3A), suggesting an additive effect. The combination of Ni-EPL ($P = 0.058$) and Ni-P100 ($P = 0.068$) showed a significant interaction (Fig. 2). However, unlike the combination of Ni-PLSDA, combining Ni-EPL or Ni-P100 within the tested range cannot reduce the LM cell counts over storage time (Fig. 3B and 3C), suggesting a potential antagonistic effect. The most significant interaction of antimicrobials is between LAG and P100 ($P = 0.002$; Fig. 2). However, as shown in Figure 3D, the lowest cell counts that can be achieved by this combination is a 1-log increase from days 0 to 12 when combining high concentrations of P100 with low concentrations of LAG, which suggests an antagonistic effect between these two antimicrobials.

DISCUSSION

Importance of developing and validating an MHM. The importance of testing the efficacy of antimicrobial treatments to control pathogenic bacteria directly on the food matrix has been reported before. Soni et al. *(26)* tested the efficacy of Ni and LAG directly in tryptic soy broth (TSB) and on the surface of cold-smoked salmon. Results showed a 4.0 log CFU LM reduction after 24 h in TSB treated with 500 ppm of Ni. TSB treated with 200 ppm of LAG completely inhibited LM after 24 h. However, when the same treatments were added to the surface of cold-smoked salmon, samples showed reductions between 2 to 3 log CFU LM after 24 h of treatment. Although some literature supports an increased MIC in food compared with broth, the opposite has also been reported. For example,

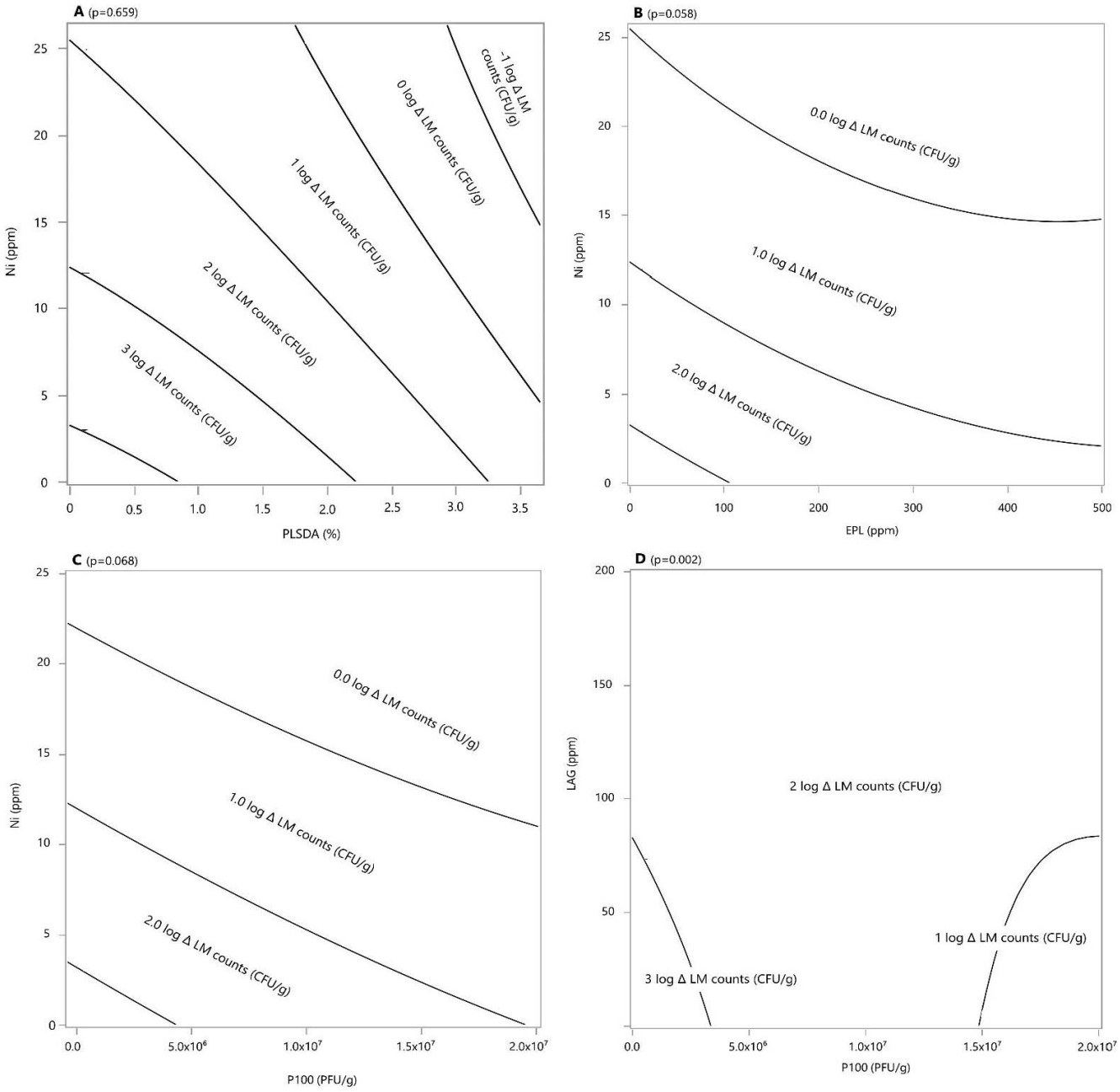

FIGURE 3. *Contour plots for the interactions between antimicrobials to change the cell counts of LM cocktail after 12 days of storage at 4°C on the MHM RSM. (A) The nonsignificant interaction (i.e., additive effect between Ni [parts per million] and PLSDA [percentage;* P *= 0.659]). (B) The modest synergistic interaction between Ni (parts per million) and EPL (parts per million;* P *= 0.058). (C) The modest syngergistic interaction between P100 (PFU per gram) and Ni (parts per million;* P *= 0.068). (D) The antagonistic interaction between LAG (parts per million) and P100 (PFU per gram;* P *= 0.002).*

Lewis et al. *(18)* determined the MIC of Ni and P100 in TSB and on coleslaw. The reported MIC for P100 and Ni on TSB was 100 multiplicity of infection and 400 ppm, respectively. However, the MIC on coleslaw of P100 and Ni was 10 multiplicity of infection and 25 ppm, respectively. Hypothesized explanations for the difference in the reported MIC might be due to storage temperature (37°C for TSB and 4°C for coleslaw), pH (7.3 for TSB and 3.9 to 4.2 for coleslaw) salt concentration, and water activity *(18)*.

Moreover, performing experiments with different antimicrobial combinations over time by using the current whole slice method can be very expensive. Martin et al. *(20)* studied the effect of LAG on frankfurters formulated with or without PLSDA on LM over 156 days. Three levels of PLSDA, two levels of LAG, and nine time points in triplicate under a full factorial design lead to almost 160 samples. The experiment was carried out only once because of the significant expense of manufacturing the required number of frankfurters for the experimental design. Therefore, we implemented and validated an MHM to improve high-throughput systems to potentially identify effective antimicrobial combinations against LM in a time- and cost-effective manner. The developed MHM mimics storage and formulation conditions commonly found in

commercially available deli hams, which are open, sliced, and manipulated after thermal processing. Validating food models for other RTE meat products, such as sausages, frankfurters, or salmon, could be done in a similar manner as the one presented in this study. Different packaging technologies, such as vaccum packaging or modified atmospheres can also be studied by using our MHM as a starting point.

RSM as a tool to screen for promising combinations. Screening methods are often used to identify promising new antimicrobials or combinations of effective antimicrobials as part of a hurdle technology approach *(1, 17, 21, 35)*. However, statistical analytical tools such as RSM are promising alternatives for time- and cost-effective screening techniques. RSM has been reported before by Borges et al. *(5)* to optimize high pressure treatments and time to control chemical and microbial spoilage on fermented sausages. Dussault et al. *(9)* applied RSM to study two-way interactions between organic acids, Ni combined with hop alpha acids, sodium chloride, and pH on ham by using the ham slice method. In the present study, a validated MHM and RSM combined was used as a potential screening method. The experimental design presented in this study can be used as an iterative tool, applying augmented designs to our already existant design of the experiment, which allows the addition of new antimicrobials without having to conduct all the experiments again. It is possible to include only the experiments not present in the original design (using five antimicrobials) to a new experimental design (using six or more antimicrobials). Additionally, the prediction formula can be used to predict the LM cell counts of any two-way combination of antimicrobials within the tested limits. Using the proposed RSM to screen novel antimicrobials, such as essential oils, can lead to the optimization of antimicrobial treatments by using the prediction formula to control LM in RTE meat products.

The most effective two-way interaction to reduce the cell counts of LM on the MHM was Ni-PLSDA. A similar effect between these antimicrobials on cold-smoked salmon has been reported before *(16, 28)*. The pore-forming action of Ni can potentiate the action of PLSDA by facilitating the diffusion of this organic salt into the cytoplasmic space *(16)*. The combined effect of Ni-EPL has been reported to be effective on minced tuna and salmon roe *(27)*. However, the effectiveness of EPL highly depends on the food matrix. High protein contents negatively affect the activity of EPL *(12)*. Our results did not show any synergistic effect between Ni and P100. However, Lewis et al. *(18)* reported a slight synergistic effect on coleslaw. This difference may be due to the different semiliquid nature in which coleslaw was analyzed, compared with the porous surface of the ham. These differences affect the diffusion of phages, which ultimately affects the ability of the phage to bind to its bacterial target *(13)*. Other authors have reported Ni-P100 to be an effective combination to reduce LM cell counts on RTE meat products *(11, 26)*. Our results showed a potential antagonistic effect between LAG and P100. The LAG-P100 combination has not been effective against LM when tested in other RTE products, such as cold-smoked salmon *(26)* and Queso Fresco *(24)*. Other studies have reported an effect of LAG on LM cell counts within the first hours after treatment. However, LAG allows LM cells to regrow over time *(20)*. A possible explanation for the potential antagonistic effect of the LAG-P100 combination is the inability of P100 to bind to the remaining LM cells due to cellular debris caused by the initial bactericidal effect of LAG on the surface of ham.

Studying the effect of antimicrobial treatments directly on food products in a time- and cost-effective way was achieved in the present study. A MHM was developed and validated against the ham slice method. Additionally, RSM was used as a screening tool to effectively identify antimicrobial treatments that should be further studied. The present study aimed to establish an RSM by using the MHM. For this purpose, GRAS antilisterial treatments were used. The proposed RSM can be used to test novel antimicrobials, such as essential oils. RSM is an alternative to full factorial designs that result in a large number of treatments when many independent variables are being screened. Traditional full factorial designs and the ham slice method result in hard, expensive, and time-consuming experiments. The combination of RSM and the MHM is a time- and cost-effective screening tool that identifies effective two-way antimicrobial combinations that can be further studied by using the ham slice method. Further studies will focus on validating the results from the RSM on ham manufactured with different antimicrobial concentrations, as well as validating the MHM against different types of RTE meat products packaging technologies.

ACKNOWLEDGMENTS

The research was supported by the U.S. Department of Agriculture Cooperative State Research, Education, and Extension Service Hatch Projects ILLU-698-903 and ILLU-698-339. We thank the University of Illinois Meat Science Laboratory for help in the production of the ham.

SUPPLEMENTAL MATERIAL

Supplemental material associated with this article can be found online at: https://doi.org/10.4315/JFP-20-435.s1

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