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Research Paper

Application of Encapsulated and Dry-plated Food Acidulants to Control *Salmonella enterica* in Raw Meat-based Diets for Dogs

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ABSTRACT

There is an increasing demand for raw meat-based diets (RMBDs) for dogs, but these foods cannot be heat-pasteurized. Thus, the objective of this study was to evaluate the antimicrobial efficacy of encapsulated and dry-plated glucono delta lactone (GDL), citric acid (CA), and lactic acid (LA) when challenged against *Salmonella enterica* inoculated in a model raw meat-based diet (RMBDs) for dogs. Nutritionally complete, raw diets were formulated with different levels (1.0, 2.0 and 3.0% (w/w)) of both encapsulated and dry-plated GDL, CA, and LA with both the positive (PC) and the negative controls (NC) without acidulants. The diets were formed into patties of ~100 g and inoculated with 3-cocktail mixtures of *Salmonella enterica* serovars, excluding the NC to achieve a final concentration of ~6.0 Log CFU/patty. Microbial analyses were performed on the inoculated diets and survivors of *S. enterica* enumerated. Both encapsulated and dry-plated CA and LA had higher log reductions compared to GDL ($P < 0.05$). However, encapsulated CA and LA at 1.0% (w/w) exhibited higher log reductions ($P > 0.05$) and preserved product quality compared to the dry-plated acidulants at 1.0%. We concluded that 1.0% (w/w) of encapsulated citric or lactic acids could be successfully applied as an antimicrobial intervention in raw diets for dogs.

Most domesticated dogs in developed countries are currently fed with commercial diets that are mostly produced through extrusion, canning, or baking processes. There is a newer dietary trend in which some pet owners prefer their animals to consume diets that are manufactured with "natural", raw, or minimally processed ingredients and preferably without heat pasteurization or cooking (Bottari et al., 2020; Buff et al., 2014; Davies et al., 2019; Fredriksson-Ahomaa et al., 2017). Raw pet food is perceived as a "healthier" alternative to commercially heat-treated diets (Fredriksson-Ahomaa et al., 2017; Freeman et al., 2013). This is because high-temperature processing is associated with degradation of heat labile nutrients and the formation of harmful, undesirable compounds through complex Maillard reactions (Delgado-Andrade et al., 2012; Förster et al., 2005; Sandri et al., 2016; van Rooijen et al., 2014), reinforcing the arguments for the proponents of raw meat-based diets (RMBDs). The Association of American Feed Control Officials (AAFCO) (Association of American Feed Control Officials (AAFCO), 2020), defines RMBDs as pet foods that cannot be heat processed, rendered, hydrolyzed, or fermented therefore making the process of pasteurizing them arduous.

Typically, RMBDs are formulated with animal meats such as chicken, turkey, beef, duck, veal, horse, lamb, and venison, and organ meats like heart, liver, and rumen. These are supplemented with bones, fish, dairy products, vegetables, fruits, and plant oils and may be fortified with vitamins and trace minerals. Given the production of RMBDs precludes use of heat to aid pathogen mitigation, and the ingredients included are known to be inherently contaminated with foodborne pathogens, there is a high probability that these diets will test positive upon microbial analysis. As such, there is concern that RMBDs are potential vehicles for the transmission of enteric foodborne pathogens to animals or humans through handling and cross-contamination because they lack a "true" kill-step and the alternative antimicrobial intervention strategies have been inefficient and costly (Bojanić et al., 2022; Bottari et al., 2020; Nüesch-Inderbinen et al., 2019; Soffer et al., 2016). Foodborne pathogens such as *Salmonella* spp., *Campylobacter jejuni*, *Listeria* spp., *Yersinia* spp. and enterohemorrhagic *Escherichia coli* have been isolated from commercial RMBDs (Bojanić et al., 2022; Domesle et al., 2021; Nüesch-Inderbinen et al., 2019; Soffer et al., 2016). Although infection with enteric foodborne pathogens might leave healthy dogs asymptomatic, studies have

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shown that these pets shed pathogens into the environment; especially if their fecal matter is not appropriately disposed (Acuff et al., 2021; Anturaniemi et al., 2019; Morley et al., 2006; Runesvärd et al., 2020; Viegas et al., 2020).

Human foodborne disease outbreaks resulting from contaminated pet foods have been reported. For example, the *Salmonella* Schwarzengrund outbreak that occurred between 2006 and 2007 made at least 70 people ill in 19 states across the United States (Centers for Disease Control and Prevention (CDC), 2008). An epidemiologic investigation conducted by Hassan et al. (2019) for the years 2017–2019 found a correlation between ground turkey and raw turkey pet products with a multi drug-resistant outbreak of *Salmonella* Reading that infected 356 people in 42 states, including District of Columbia (DC). Case counts regarding transmission of foodborne pathogens from RMBDs to humans are likely underestimated (Finley et al., 2006; Nüesch-Inderbinen et al., 2019) and thus cost effective and efficient antimicrobial interventions to aid pathogen mitigation in raw diets need to be investigated.

Food acidulants like glucono delta lactone (GDL), citric, and lactic acids are generally regarded as safe (GRAS) and have exhibited potent antimicrobial activity against many foodborne pathogens inherently found in meat and poultry products (Mani-López et al., 2012). However, the utilization and direct application of food acidulants such as citric and lactic acids in meat and poultry products have been impeded by the negative effects observed on finished product quality and sensory properties as these acids can cause lipid oxidation, discoloration (from pink to gray), and syneresis (Kiprotich et al., 2021; Wang et al., 2015). However, encapsulation of food acidulants with edible vegetable oil coatings promises to mitigate the challenges caused by direct application of these acidulants in meat and poultry products. Encapsulation of food acidulants allows for a delayed release of the acid into the product, reduces the impact of the acid on product quality and sensory attributes, while retaining its antimicrobial efficacy (Kiprotich et al., 2021). While there is a large body of published reports on the use of food acidulants to control *Salmonella enterica*, we have no knowledge of published work comparing the antimicrobial efficacy and effect on pH of encapsulated and dry-plated acidulants in raw pet food. Therefore, the primary objective of this study was to evaluate the antimicrobial efficacy of encapsulated and dry-plated GDL, citric, and lactic acids when challenged against *Salmonella enterica* inoculated in a model raw diet for dogs. The secondary objectives were to calculate the decimal reduction times (D-values) of the food acidulants *in-vitro* and when included in the raw diets and challenged against *S. enterica* serovars. The other objective was to compare and monitor pH changes in the raw diets when encapsulated or dry-plated acidulants were used as antimicrobials in raw diets for dogs.

Materials and methods

Bacterial strain and culture conditions

Three frozen (−80°C) isolates belonging to different serotypes of *Salmonella enterica* (Heidelberg ATCC 8326, Typhimurium ATCC 14802, and Enteritidis ATCC 13076) were obtained from the culture collection in the Feed Toxicology and Microbiology Laboratory at Kansas State University. The cultures were stored frozen in a mixture of Tryptic Soy Broth (TSB) (Difco; Becton, Dickinson and Company, Sparks, MD) and glycerol. The ratio of the quantity of TSB to glycerol in the vials storing the frozen cultures was 7:3. The vials containing the frozen cultures were thawed and then transferred to fresh TSB and incubated at 37°C for 24 h for activation. For preparation of working cultures, the activated cultures were transferred into TSB twice consecutively within 24 h following incubation at 37°C. To prepare the inoculum, 10 mL of each individual *S. enterica* serovar suspended in TSB was centrifuged (5,000 × *g*, 10 min, 20°C) using a Sorvall X1R centrifuge (Thermo Scientific). The cells were then suspended in 10 mL of 0.85% (w/v) NaCl (saline) to obtain a final viable cell concentration of 9 Log CFU/mL. To make a 3-cocktail mixture of *S. enterica* serovars, all the cells suspended in saline were mixed in a 50-mL conical tube.

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration assay

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of only dry-plated GDL (GDL-RW), citric acid (CA-RW), and lactic acid (LA-RW) exposed to planktonic cells of *S. enterica* were evaluated using the broth microdilution assay. For the MIC, 0.2 mL (40% (w/v)) of the treatment solutions of GDL-RW, CA-RW, and LA-RW were pipetted into the first well of each row of the microtiter plate to reach a final concentration of the food acidulants in the treatment solutions of 20% (w/v) upon addition of the inoculum. The remaining 11 wells were filled with 0.1 mL of sterile distilled water. A 1:2 serial dilution of the treatment solution from the first well was performed till the 11th well and the 0.1 mL solution from the 11th well was discarded. The final treatment concentrations in the wells were 20, 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156, and 0.078%. Then 0.1 mL of the three-strain working culture was added into each well. Negative controls contained sterile distilled water and TSB without inoculum. The positive control contained 0.1 mL TSB and 0.1 mL of *Salmonella* inoculum. The microtiter plate containing the least concentration of the treatment solution and did not show any visible signs of bacterial growth, i.e., turbidity was considered the MIC. For MBC, 0.1 mL of sample from each well was plated on to tryptic soy agar (TSA) for enumeration of bacterial colonies at each concentration of the treatments. The concentration that had a 3-Log reduction from the initial inoculum was considered the MBC.

Preparation and inoculation of acidified sterile distilled water (in-vitro time-kill assay)

For the in-vitro time-kill assay, sterile distilled water (SDW) was used to dissolve dry-plated acidulants, CA-RW, LA-RW, and GDL-RW to prepare treatment solutions at the following concentrations, 1.0, 3.0, and 5.0% (w/v). Treatment solutions (20 mL) were aseptically dispensed to 50-mL conical tubes and then 0.2 mL of a 3-serotype cocktail of *Salmonella enterica* inoculated into the treatment solutions to achieve a final pathogen concentration of ~7.0 Log CFU/mL. Microbial analysis was then performed at the following time intervals of 0, 15, 25, 35, and 45 min. At each time interval, 1.0 mL of inoculated treatment solutions were obtained from the conical tubes and then dispensed to 9.0 mL of buffered peptone water (BPW) to neutralize the acid and stop microbial inactivation. Serial dilutions were then performed and 0.1 mL aliquots from neutralized treatment solutions were then plated on tryptic soy agar (TSA) and incubated at 37°C for 48 h when the colonies were enumerated.

Preparation of raw meat-based pet food

A nutritionally complete model raw pet food was prepared using an in-house formula under sanitary conditions in a food-grade laboratory to minimize contamination. Ingredients turkey, sweet potato, chicken liver, carrots, and apples were purchased from a local grocery store in Manhattan, Kansas, weighed and ground together to form a batter. The encapsulated and dry-plated Petshure™ GDL, citric, and lactic acids were obtained from Balchem Corporation for research purposes. The encapsulated acidulants were coated in edible oil films to form powdery flakes, such that 100 g of acidulant contained only 30% (w/w) of either CA, LA, or GDL. The dry-plated acidulants were described as “raw” as they were not coated in film and 100 g of the crystalline acidulants only contained 60% (w/w) of either CA, LA, or GDL acidulants within their matrix.

To prepare treatments, three levels of encapsulated and dry-plated acidulants at 1.0, 2.0, and 3.0% (w/w) inclusion levels were added individually to 2.0 kg of ground meat batter and mixed, including a negative and positive control that did not contain any acidulants for a total of N = 20 treatments. The batter was mixed thoroughly and then shaped to form patties of approximately 100 g. The patties were individually stored in sterile, self-sealing stomacher bags (VWR), and refrigerated for 2 h at 4°C prior to inoculation. Before inoculation, patties from each treatment including the negative and positive controls were analyzed for presence of background *Salmonella.*

Inoculation of raw meat-based pet food

All the patties that contained acidulants including the positive control were gently removed from the stomacher bags and placed in sterile petri dishes in a biohazard hood and inoculated with 0.1 mL of the 3-cocktail mix of *Salmonella enterica* serovars to achieve a final concentration of ~6.0 Log CFU/patty. A sterile L-rod was used to spread the inoculum on the flat top of the patty. The patties were then held in the biohazard hood for 30 min to allow for pathogen attachment before being transferred to new sterile stomacher bags and put back in the refrigerator at 4°C. The negative control without any acidulant was not inoculated as they were for testing growth of background *Salmonella* during the study.

Microbiological analysis

Microbial analyses for all the treatments including the negative control were performed on days 1, 4, 7, 10, 13, 16, 19, and 22 to mimic the anticipated shelf-life for refrigerated RMBDs. For analysis, 100 mL of BPW was added to the stomacher bags and pummeled with a stomacher machine (Seward) at medium speed for 1 min. For background *Salmonella* analysis following homogenization in the stomacher, the negative control samples were serially diluted in 0.1% peptone (Difco; Becton, Dickinson and Company, Sparks, MD) and appropriate dilutions of 0.1 mL aliquots spread plated on Xylose-Lysine-Tergitol 4 (XLT-4) agar. The above procedure was repeated for all the remaining 19 treatments in the study. The plates were incubated at 37°C for 48 h, and then colonies of *S. enterica* were enumerated and log reductions calculated by subtracting the initial number of *S. enterica* pathogens from the survivors after exposure to food acidulants during storage. The initial inoculum (Log CFU/patty) was determined through microbial analysis of fresh negative control patties after 30 min of attachment in the biohazard hood.

Determination of D-values

The *D*-values (time of exposure to food acidulant that results in 90% reduction in viable counts of *Salmonella enterica* serovars) were determined by plotting the log number of survivors per sample (inoculated patties) vs. exposure time (days) using Software (Microsoft Inc.). Using linear regression analysis, the line of best fit for each set of data was determined. The D-value was evaluated by calculating the negative reciprocal of the slope of the regression line.

Measurement of pH

The pH of individual patties was measured on the same days microbial analyses were performed i.e., on days 1, 4, 7, 10, 13, 16, 19, and 22. The measurements were taken by inserting a probe into meat patties using an Apera PH700 Benchtop pH Meter (Apera Instruments, LLC) with a stainless-steel electrode.

Statistical analysis

The microbial challenge study (inoculation and enumeration of *S.* enterica in RMBD) was conducted as a (6 × 3) + 2 factorial arrangement of treatments (six acidulants, three treatment levels and two controls, both positive and negative) for N = 20, and experiments were replicated thrice. The *in-vitro* time-kill experiment was conducted as a (3 × 3) + 1 factorial arrangement of treatments. Two-way analysis of variance (ANOVA) was used to determine treatment levels with significant log reductions in the patties and the treatment solutions that were treated with different food acidulants at varying inclusion levels over time. One-way ANOVA was used to separate the log reduction and D-value means of the RMBD patties that were treated with food acidulants after 22 days. The means obtained from the log reduction and D-values of the patties treated with food acidulants were evaluated for significant differences at a 5% significance between the treatment levels using Tukey's test ($P \leq 0.05$), respectively. For statistical purposes, the D-values from the control experiments (challenge study and *in-vitro* assay) were excluded from models as they were outliers and increased error variance and reduced the power of the tests. Variability in the data is expressed as the standard error of the means (SEM). The MIC and MBC experiments were replicated thrice to ensure the results consistency. The changes in mean pH over time and standard deviation were calculated and presented in figure format. Data were analyzed using JMP Pro version 16.0 statistical software (SAS Institute, Inc., Cary, NC).

Results

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The dry-plated GDL, lactic and citric acids had their initial concentrations ranging from 0.156% to 20% (w/v) in sterile distilled water. The MIC was determined in the wells that showed no turbidity (no microbial growth). For lactic and citric acids, their MIC was 0.313%, whereas the MIC of GDL was 0. 625% and inhibited growth of *S. enterica.* The MBC of lactic and citric acid was 1.25%, while that of GDL was 5.0% with these concentrations of acidulants resulting in a ~3.0 Log CFU/mL reduction of the initial viable counts of *S. enterica.*

Viability of S. enterica in acidified sterile distilled water (In-vitro time-kill assay)

The dry-plated acidulants GDL-RW, CA-RW, and LA-RW were dissolved in sterile distilled water (SDW) at different concentrations (1.0, 3.0, 5.0 (w/v)) to form acidified solutions which were inoculated. The viable counts of *S. enterica* in SDW without any acidulant was ~7.0 Log CFU/mL. The log counts of survivors of *S. enterica* over time when inoculated into SDW containing food acidulants and enumerated on TSA media are provided in Figure 1. There was no change in the survivor counts of *S. enterica* in the negative control (NC) (~7.2 ± 0. 5 Log CFU/mL) as the SDW did not contain any acidulant. The *S. enterica* survivors inoculated in SDW containing GDL-RW were consistently higher ($P < 0.05$) compared to those exposed to LA-RW and CA-RW. The counts of *S. enterica* declined ($P < 0.05$) as the concentrations of both LA-RW and CA-RW increased from 1.0% to 5.0% (w/v); whereas there was no decrease ($P > 0.05$) in the number of counts for *S. enterica* exposed to GDL-RW despite increases in concentration of GDL from 1.0% to 5.0% (w/v). *Salmonella enterica* was below detectable limits when exposed to SDW containing both LA-RW and CA-RW at 5.0% (w/v) after 35 min. The NC had the least log reduction ($P < 0.05$) of ~0.5 Log CFU/mL compared to the rest of the treatments. There was no difference ($P > 0.05$) in the total log reductions observed when *S. enterica* was exposed to all the different levels of GDL-RW. Dry-

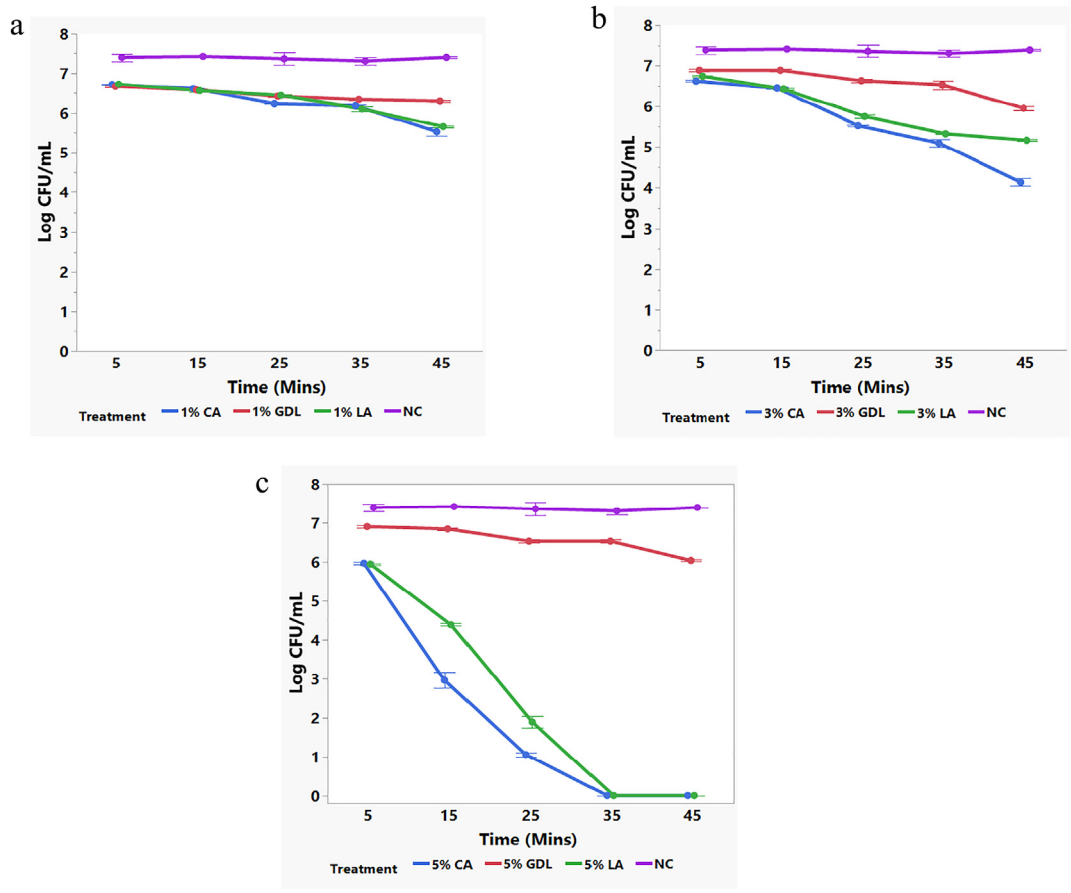

Figure 1. Log CFU/mL of survivors of *Salmonella enterica* serovars when planktonic cells are exposed to treatment solutions of dry-plated food acidulants (RW), GDL-RW, CA-RW and LA-RW, respectively at 1.0% (A), 3.0% (B), and 5.0% (C) (w/v) for 45 min (*in-vitro* assay) including the NC (negative control) whereby sterile distilled water was inoculated with *Salmonella* as a control. The bars on the figures represent the standard deviation of the means.

plated citric and lactic acids at concentrations of 1.0% and 5.0% (w/v) had no differences in log reduction ($P > 0.05$) but were different ($P < 0.05$) at 3.0% (w/w). Overall, CA-RW exhibited the strongest bactericidal effect of the three dry-plated acidulants, followed by LA-RW and GDL-RW that had the least antimicrobial effect against *S. enterica* inoculated in the treatment solutions.

Survival of Salmonella enterica on raw meat-based patties for dogs

No *Salmonella* was detected on the noninoculated NC samples for the entirety of the study. The numbers of survivors of *S. enterica* that were inoculated on meat-based patties were enumerated on XLT-4 agar and are represented in Figure 2**.** The initial viable counts of *S. enterica* was ~6.02 Log CFU/patty after inoculation and a 30-min period of attachment at room temperature (20 ± 2°C). All treatments resulted in a reduction of viable ($P < 0.05$) counts of *S. enterica* compared to the PC which were not treated with acidulants but were inoculated with pathogens.

Dry-plated lactic and citric acids at 2.0 and 3.0% (w/w) resulted in higher ($P < 0.05$) log reductions compared to the encapsulated acids at the same concentration levels. However, at 1.0% (w/w), encapsulated GDL, lactic, and citric acids had higher log reductions ($P > 0.05$) than similar dry-plated acidulants at the same concentration. When Log reduction comparisons across different treatments at 1.0% (w/w) were performed, LA-ENC had the highest numerical reduction compared to LA-RW, CA-RW, CA-ENC, GDL-RW, and GDL-ENC though not significant. At 1.0% (w/w), encapsulated acidulants had higher log reductions ($P > 0.05$), but as concentrations increased to 2.0–3.0% (w/w), the dry-plated acidulants had significant log reductions compared to encapsulated acids at the same concentrations. Overall, both GDL-RW and GDL-ENC had significantly lower log reductions compared to encapsulated and dry-plated lactic and citric acids. Table 1 shows a summary of all the log reductions observed in all treatments after 22 days when *S. enterica* was inoculated on meat patties treated with encapsulated and dry-plated GDL, citric, and lactic acids at inclusion levels of 1.0, 2.0, and 3.0% (w/w).

Decimal reduction times (D-values)

Table 2 shows the effect of the food acidulants at different inclusion levels in treatment solutions prepared from dissolving the dry-plated acids in sterile distilled water on the D-values in minutes of planktonic cells of *S. enterica.* There was a difference ($P < 0.05$) in the D-values obtained from CA-RW and LA-RW compared to the GDL-RW at all concentrations. There was no difference ($P > 0.05$) between the D-values obtained from GDL-RW across all concentrations of 1.0, 3.0, and 5.0% (w/v). There was also no difference ($P > 0.05$) in the D-values obtained from CA-RW and LA-RW at 1.0% and 5.0% (v/w) although differences between these two acidulants were observed at 3.0% whereby citric acid had a lower D-value. Increments in the concentration of treatment solutions from 1.0% to 5.0% resulted in a significant reduction ($P < 0.05$) in D-values for both citric and lactic acids but not GDL for the planktonic cells.

Table 3 shows the decimal reduction times in days (D-values) for *Salmonella enterica* serovars inoculated and attached to raw meat-based pet food treated with food acidulants, GDL, citric, and lactic

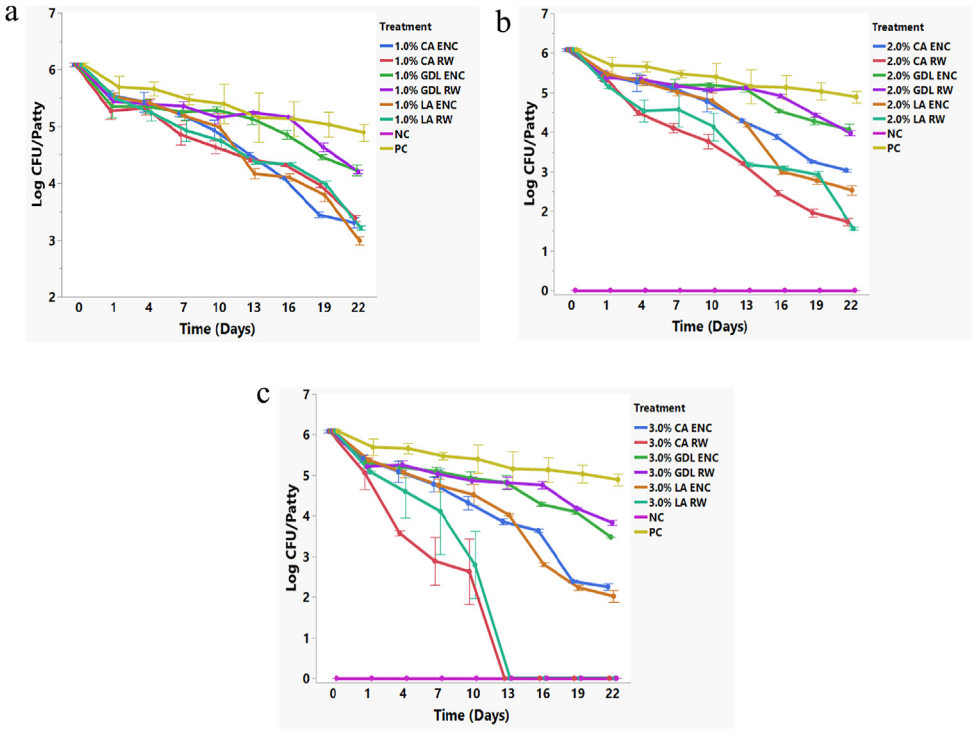

Figure 2. Comparison of Log CFU/patty survivors of *Salmonella enterica* serovars artificially inoculated in raw meat patties treated with encapsulated (ENC) and dry-plated (RW) GDL, citric and lactic acids at 1.0% (**A**), 2.0% (**B**) and 3.0% (**C**) (w/v) inclusion levels, for up to 22 days (microbial challenge study). The NC (negative control) was to monitor background *Salmonella* whereas the PC (positive control) were patties inoculated with *Salmonella* but contained no acidulants. The bars on the figures represent the standard deviation of the means.

Table 1
The total log reduction of *Salmonella enterica* serovars that were inoculated in raw meat-based patties for dogs after 22 days of exposure to different concentrations (w/w) of encapsulated and dry-plated GDL, citric, and lactic acids

Treatment	Log CFU/patty Reduction[1]	SEM
PC	1.19^{A}	0.116
1.0% GDL-RW	1.88^{B}	0.019
1.0% GDL-ENC	1.86^{B}	0.061
1.0% LA-RW	2.88^{C}	0.07
1.0% LA-ENC	3.10^{E}	0.104
1.0% CA-RW	2.70^{CD}	0.083
1.0% CA-ENC	2.78^{CD}	0.12
2.0% GDL-RW	2.11^{BC}	0.038
2.0% GDL-ENC	2.02^{BC}	0.109
2.0% LA-RW	4.53^{E}	0.007
2.0% LA-ENC	3.55^{D}	0.145
2.0% CA-RW	4.35^{E}	0.12
2.0% CA-ENC	3.05^{C}	0.07
3.0% GDL-RW	2.25^{B}	0.033
3.0% GDL-ENC	2.61^{CD}	0.033
3.0% LA-RW	6.09^{H}	0.033
3.0% LA-ENC	4.07^{G}	0.175
3.0% CA-RW	6.09^{H}	0.033
3.0% CA-ENC	3.84^{FG}	0.109

Means with different superscript (A, B, C, D, E, F, G, H) are considered different at $P < 0.05$.
[1]Total log reduction is obtained by subtracting the initial inoculum from the final Log CFU counts that had been obtained on day 22.
*GDL- Glucono delta lactone, CA- Citric acid, LA- Lactic acid, RW- Dry-plated acidulant, ENC-encapsulated acidulant.

acids at inclusion levels of 1.0, 2.0, and 3.0% and stored at 4°C. At 1.0% (w/v), both encapsulated and dry-plated citric and lactic acids had significantly ($P < 0.05$) lower D-values compared to GDL, but

Table 2
Decimal reduction times in minutes (D-values) for *Salmonella enterica* planktonic cells suspended in treatment solutions containing acidulants GDL, CA-RW, and LA-RW at 1.0%, 3.0% and 5.0% (*in-vitro*).

Treatment	D-Value (Min)	SEM
1.0% GDL	49.85^{A}	0.717
1.0% LA	35.55^{C}	0.299
1.0% CA	35.56^{C}	1.328
3.0% GDL	48.89^{A}	1.128
3.0% LA	23.56^{D}	0.093
3.0% CA	15.85^{E}	0.438
5.0% GDL	45.10^{B}	0.807
5.0% LA	6.15^{F}	0.016
5.0% CA	6.72^{F}	0.063

Means with different superscript (A, B, C, D, E, F) are considered different at $P < 0.05$. The data used to calculate the D-values were derived from bacterial counts of survivors of *S. enterica* inoculated into treatment solutions for up to 45 minutes.
*GDL- Glucono delta lactone, CA- Citric acid, LA- Lactic acid, RW- Dry-plated acidulant, ENC-encapsulated acidulant.

exhibited no significant difference in the D-values observed from both CA and LA. Also, the encapsulated acidulants at 1.0% exhibited lower D-values than the dry-plated acids, but no significant difference was observed ($P > 0.05$). At 2.0% and 3.0% inclusion levels in the raw diets, dry-plated acidulants such as CA-RW had significantly ($P < 0.05$) lower D-values compared to CA-ENC, whereas no difference ($P > 0.05$) was observed in the D-values obtained from LA-RW and LA-ENC. The raw diets treated with CA-RW and LA-RW at 3.0% also had the least D-values of the study and therefore achieved a 90% reduction in the initial population of *S. enterica* pathogens in approximately 3.4 days.

Table 3
Decimal reduction times in days (D-values) for *Salmonella enterica* serovars inoculated to raw meat-based pet food treated with dry-plated or encapsulated food acidulants, GDL, citric, and lactic acids at inclusion levels of 1.0, 2.0, and 3.0% and stored at 4°C.

Treatment	D-Value (Days)	SEM
1.0% CA-ENC	8.3^{EFG}	0.015
1.0% CA-RW	10.2^{DE}	0.197
1.0% GDL-ENC	16.0^{AB}	1.736
1.0% GDL-RW	16.9^{A}	1.270
1.0% LA-ENC	8.1^{EFG}	0.073
1.0% LA-RW	9.4^{DEF}	0.151
2.0% CA-ENC	7.7^{FGH}	0.152
2.0% CA-RW	5.3^{IJ}	0.210
2.0% GDL-ENC	13.5^{C}	1.161
2.0% GDL-RW	13.9^{BC}	0.011
2.0% LA-ENC	6.2^{GHI}	0.120
2.0% LA-RW	6.0^{GHI}	0.293
3.0% CA-ENC	6.1^{GHI}	0.108
3.0% CA-RW	3.53^{J}	0.170
3.0% GDL-ENC	10.8^{D}	0.526
3.0% GDL-RW	13.4^{C}	1.122
3.0% LA-ENC	$5.6H^{IJ}$	0.120
3.0% LA-RW	3.29^{J}	0.187

Means with different superscript (A, B, C, D, E, F) are considered different at $P < 0.05$. The data used to calculate the D-values were derived from bacterial counts of survivors of S. enterica inoculated into raw meat-based patties formulated with different inclusion levels of food acidulants for a period of up to 22 days.
*GDL- Glucono delta lactone, CA- Citric acid, LA- Lactic acid, RW- Dry-plated acidulant, ENC-encapsulated acidulant.

pH of raw meat-based patties

The acidulants at the three concentrations (1.0, 2.0 and 3.0% (w/w) impacted the pH of the meat patties differently (Fig. 3). In the positive and negative control patties, the pH gradually dropped, leveled, and then started rising toward the end of the study. A rapid drop in pH was observed in the meat patties that were treated with LA-RW, CA-RW, and GDL-RW. However, the patties that were treated with encapsulated acids had a gradual drop in pH compared to the dry-plated acidulants. The NC and PC had a higher ($P < 0.05$) pH compared to the patties that were treated with both dry-plated and encapsulated acids after 22 days.

There was a sharp decline ($P < 0.05$) in the pH of the patties treated with dry-plated acidulants after day 1 compared to the patties treated with encapsulated acids and the controls. However, the decline in pH between the control patties and those treated with encapsulated acids was not significant after 1 day. There were no declines in pH observed for the patties that had been treated with the same encapsulated acid even when the concentration levels were increased from 1.0% to 3.0% (w/w). For instance, there were no declines ($P > 0.05$) in pH of the patties treated with LA-ENC at 1.0, 2.0, or 3.0% (w/w); moreover, a similar trend was observed in GDL-ENC and CA-ENC at the same concentrations. Increasing the concentration of dry-plated acidulants (CA-RW, LA-RW, and GDL-RW) from 1.0% to 3.0% (w/w) did not result in any further decline ($P > 0.05$) in pH after day 1, as there was a slight increase observed. There was an increase in pH observed in the patties that were treated with dry-plated acidulants after 4 days though it was not significant among the different concentration levels within treatments. Overall, there was a difference in the rate of pH decline for the patties that were treated with either encapsulated or dry-plated acidulants.

Discussion

The MIC test was performed to investigate the minimum concentrations of food acidulants that would inhibit microbial proliferation *in-vitro*. The MIC in this case was to provide an insight into the antimicrobial efficacy of the individual dry-plated acidulants (LA-RW, CA-RW

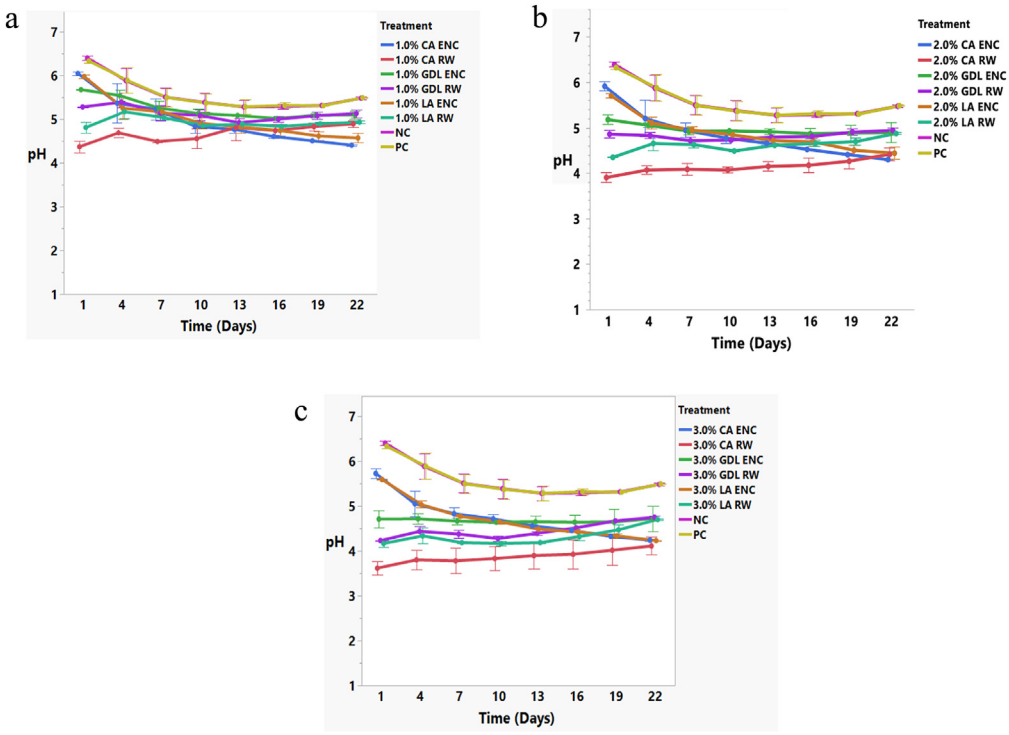

Figure 3. Comparison of changes in pH of raw meat-based patties treated with different levels of encapsulated (ENC) and dry-plated (RW) GDL, citric, and lactic acids at 1.0% (**A**), 2.0% (**B**) and 3.0% (**C**) (w/v) inclusion levels, compared with negative (NC) and positive control (PC) patties without acidulants during a 22-day period of storage at 4°C. The bars on the figures represent the standard deviation of the means.

and GDL-RW). For instance, lactic and citric acids had a similar and lower MIC compared to GDL. When exposed to CA-RW and LA-RW, the MIC was 0.313%, which was slightly lower than 0.5% reported by Wang et al. (2015). The MBC was intended to provide the lowest concentration of food acidulant capable of a 3.0 Log CFU/mL pathogen reduction. The MBC for lactic and citric acids were more bactericidal compared to GDL. This is probably because GDL is mainly used as an acidifier, which upon dissolution in water becomes partially hydrolyzed to form a weak acid, gluconic acid with minimal antimicrobial activity (Zhou et al., 2020).

The purpose of the *in-vitro* time-kill assay was to investigate the antimicrobial efficacy of the different types of dry-plated acidulants against planktonic cells of *S. enterica* serovars by measuring their susceptibility against the acidulants. Lactic and citric acids exhibited more antimicrobial potency compared to GDL, even when the concentrations were increased. The log reduction of *Salmonella enterica* observed in the *in-vitro* (acidified solutions) experiments was higher compared to the reduction in raw meat patties. These results were expected as the pathogens attach to meat surfaces and exhibit more tolerance to antimicrobial agents according to Cadena et al. (2019) and Kiprotich et al. (2021). The reduced susceptibility to food acidulants may be due to biofilm formation which serves as a barrier protecting cells from direct contact with acidulants (Cadena et al., 2019; Dimakopoulou-Papazoglou et al., 2016). Furthermore, Dimakopoulou-Papazoglou et al. (2016) reported that *Salmonella enterica* rapidly produced biofilm when exposed to low-pH conditions. This was not possible for the planktonic cells in the *in-vitro* time-kill assay, as these cells were in direct contact with acidulants, resulting into more cell death. Also, the lower log reductions that were reported when *S. enterica* serovars were inoculated into patties treated with acidulants might be due to the buffering effect of the meat proteins that neutralized the acidulants, thus reducing their lethality (Kiprotich et al., 2021; Wang et al., 2015; Yeh et al., 2018)

Overall, raw meat-based patties that were treated with dry-plated acidulants at 2.0% and 3.0% had higher log reductions than patties that were treated with encapsulated acids at similar concentrations. At 1.0%, encapsulated acids had higher log reductions compared to dry-plated acidulants at the same concentrations. This might be attributed to the buffering capacity of meat proteins that neutralized the dry-plated acids, which was observed as a slight increase in pH. Encapsulated acidulants on the other hand are coated with edible vegetable films that "melt" over time when in contact with a water-based matrix, allowing gradual release of food acid into the meat product. At 2.0% and 3.0%, dry-plated acidulants resulted in significantly higher log reductions compared to the encapsulated acids at the same concentrations. The increase in the inclusion levels of acidulants resulted in syneresis, discoloration, weeping, and visible signs of mold growth. For instance, *Salmonella enterica* counts were below detectable limits at day 13, but there was evidence of visible mold growth in the patties that were treated with CA-RW and LA-RW at 3.0% (w/w). We hypothesized that the rapid decline in pH and acid shock from the dry-plated acidulants might have resulted in significant injury and death of vegetative cells of pathogenic bacteria and in this case *Salmonella enterica*, allowing acid tolerant fungi and bacteria to proliferate.

Food acids, when dissolved in water do not dissociate completely, but rather, in a pH dependent manner. Upon adding the acids to meat or poultry, the pH of the meat is lowered to a point less than or equal to the dissociation constant (pKa) of the acid, yielding an increased amount of H^+, which then inactivate bacteria (Taylor et al., 2012). The H^+ released from food acid exhibits antimicrobial activity primarily by two mechanisms: Cytoplasmic acidification by an influx of H^+ through a transmembrane gradient disrupting ATP production, regulation, and active transport, and secondly, accumulation of dissociated anions from the acidulant until toxic levels are reached and cellular metabolic machinery fails, ultimately causing cell death (Taylor et al., 2012).

The survival of *Salmonella enterica* within the matrix of the inoculated RMBDs may have been potentially affected by the metabolites produced by psychrophilic microorganisms that thrive in refrigeration temperatures and thus might have been the dominant population since the latter is a mesophile. For instance, some strains of *Pseudomonas,* a common meat spoilage microorganism, have demonstrated competitive inhibition when they were cultured under conditions of low pH and temperature (Thomas & Wimpenny, 1996). Wang et al. (2013) also reported that cell-free supernatants containing metabolites of *Pseudomonas aeruginosa* inhibited biofilm formation in a meat-bone matrix.

The D-values in minutes (Table 2) for *S. enterica* exposed to treatment solutions represent the times required for a 90% or 1.0 log inactivation of the initial viable population of the pathogen. The rapid decrease in the D-values for planktonic cells exposed to both citric and lactic acids suggest a faster rate of microbial inactivation compared to when the cells are exposed to GDL. However, the D-values in days for *S. enterica* inoculated and attached to the raw pet food are significantly higher than those observed in the *in-vitro* experiment, which is evidence of the protective and buffering capacity of meat proteins that allows these pathogens to survive for longer periods of time. The similarities in the D-values observed from citric and lactic acids would suggest similar antimicrobial mechanisms of action, though this warrants additional research.

The pH curves that were observed in the positive and negative control patties were higher (~pH 6.4) than the typical pH of meat during cold storage because the meat in this formula was obtained from turkey, which at chilling temperatures on day 0 had a pH of 6.5 as reported by Triki et al. (2018). The patties that were treated with dry-plated acids had their pH rapidly drop on day one unlike the patties that contained encapsulated acidulants. This is because the encapsulation process likely ensured gradual release of the acid into the meat matrix. However, color transformation from pink to gray was observed in the patties treated with dry-plated acids 2 h postproduction. This was unlike the controls and those treated with the encapsulated acidulants that maintained their pink color through the entirety of the study.

Despite possessing potent antimicrobial properties, food acidulants such as dry-plated lactic and citric acids may damage product color, lead to syneresis, and result in low-quality RMBDs from the shock of direct acidification. Thus, encapsulation offers alternative methods of utilizing food acidulants to enhance the safety of raw meat diets. Additional research is needed to determine how other hurdles might be used to potentiate the antimicrobial effect of encapsulated acidulants that address safety from pathogenic microbes and spoilage caused by psychrotrophic bacteria as slimy and green discolorations were observed on the raw meat patties treated with encapsulated acidulants at the end of the study.

Foodborne pathogens such as *Salmonella enterica* are common contaminants of RMBDs. Incidences of foodborne disease outbreaks and product recalls are likely to increase as more pet owners and pet food manufacturing facilities adopt and produce more of these diets. However, without efficient and relatively inexpensive means of spoilage and pathogen control, use of GRAS additives such encapsulated food acidulants may offer a more practical means of enhancing safety in RMBDs. The FDA regulations mandate that a successful pasteurization process should achieve at least a 5.0 log reduction, which was observed when dry-plated citric and lactic acids were used as antimicrobials but led to significant deterioration in product quality. Therefore, the authors advise that encapsulated lactic and citric acids at 1.0% be applied in combination with different hurdles in raw meat-based diets to meet the FDA requirements for the control foodborne pathogens like *Salmonella enterica.* The implication of this research is that safety from enteric foodborne pathogens in raw pet food can be addressed without compromising quality; however, additional

research is warranted to study the impact of acidulants on the palatability of RMBDs.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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