Meat and Muscle Biology™

Inhibition of *Clostridium perfringens* in High-Moisture, Uncured Turkey Products by Vinegar-Based Clean-Label Ingredients During Extended Cooling

Kathleen A. Glass[1]*, McKenna P. Mahnke[1,2], and Max C. Golden[1]

[1]Food Research Institute, University of Wisconsin-Madison, Madison, WI 53706, USA
[2]Nestlé Health Science, Savannah, GA 31408, USA
*Corresponding author. Email: kglass@wisc.edu (Kathleen A. Glass)

Abstract: The United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) Appendix B guidelines identify cooling options to prevent growth of *Clostridium perfringens* in uncured meat and poultry products where Phase 1 cooling (between 49 and 27°C) is limited to 1 h and continued chilling to 4°C. This study screened several commercially available vinegar-based clean-label ingredients to inhibit *C. perfringens* in a model poultry product during extension of Phase 1 cooling. Eight uncured turkey products (75% moisture, 1.5% salt, pH 6.6 and 6.2) were formulated with no antimicrobial (control) or 1% dry vinegar (DV), dry vinegar-cultured sugar (DV-CS), or dry vinegar-fruit-spice-extract (DV-FSE). The batter was inoculated with 2.5-log CFU/g *C. perfringens* spores (3-strain mixture), vacuum-packaged (25 g/pouch), cooked to 70°C, and cooled according to the following schedule: Phase 1 (48.9–26.7°C) in 2, 3, 4, or 5 h; Phase 2 (26.7–12.8°C) in 5 h; and Phase 3 (12.8–4.4°C) in 5 h (total cooling 12, 13, 14, or 15 h). Triplicate samples were enumerated after cooking and at the end of each phase; each experiment was replicated twice. As expected, *C. perfringens* grew rapidly in control samples (>1.5-log and >6-log for the 2- and 5-h extended Phase 1 cooling, respectively). In contrast, treatments containing 1% DV or DV-FSE inhibited 1-log growth at pH 6.6 and 6.2 when Phase 1 cooling was extended to 2 and 3 h, respectively. Turkey containing 1% DV-CS at pH 6.6 and 6.2 inhibited *C. perfringens* to ≤1-log when Phase 1 was extended 4 and 5 h, respectively. This study confirms that DV-based clean-label antimicrobials can be used by manufacturers to extend Phase 1 cooling of uncured meat and poultry products beyond the recommendations in the 2021 USDA-FSIS Appendix B Option 2 Stabilization Guidelines. Further research is needed to compare the efficacy of similar ingredients among various suppliers.

Key words: *Clostridium perfringens*, clean-label antimicrobials, dry vinegar, cultured sugar, cooling, USDA-FSIS Appendix B
Meat and Muscle Biology 8(1): 17657, 1–9 (2024) **doi:10.22175/mmb.17657**
Submitted 4 January 2024 Accepted 13 June 2024

Introduction

Clostridium perfringens is a ubiquitous, spore-forming bacterium that is responsible for an estimated 1 million foodborne illnesses per year (Centers for Disease Control and Prevention, 2024). *C. perfringens* grows rapidly in foods (doubling time of 7–8 min) at temperatures between 27 and 45°C (Golden et al., 2009; ICMSF, 1996), such as during poorly controlled cooling or hot-holding (Centers for Disease Control and Prevention, 2024; Wittry et al., 2022). When an individual consumes a product containing high populations of *C. perfringens* (10^6 cells), the cells then release enterotoxin during sporulation in the intestine (toxicoinfection) causing lower gastrointestinal symptoms (Labbé and Juneja, 2013; U.S. Food and Drug Administration, 2013).

The United States Department of Agriculture Food Safety and Inspection Service (FSIS) assumes a base level of 10^4 presumptive vegetative cells and spores of *C. perfringens* in the raw product (U.S. Department of Agriculture Food Safety and Inspection Service,

2001), although more recent surveys suggest populations of less than 2-log CFU/g in raw product (Kalinowski et al., 2003; Taormina et al., 2003) and less than 1-log CFU/g in finished product (U.S. Department of Agriculture Food Safety and Inspection Service, 2023). However, the cooling guidelines are designed to limit growth to no greater than a 1-log increase, regardless of formulation, as a means to ensure that foods contain levels that are below populations known to cause infection. (U.S. Department of Agriculture Food Safety and Inspection Service, 2021; U.S. Department of Agriculture Food Safety and Inspection Service, 2023).

The 1999 version of Option 2 Appendix B Stabilization Guidelines for uncured meat and poultry products stated that the cooling process should begin within 90 min of cooking and that all product needs to be cooled to 12.7°C in no more than 6 h (U.S. Department of Agriculture Food Safety and Inspection Service, 1999). This cooling rate was considered to have a low margin of safety but reflected cooling capabilities for large-diameter meats, which are thermodynamically difficult to cool quickly, with internal temperatures of large products potentially taking 3–4 h to cool to 26.7°C under commercial conditions (personal communication, North American Meat Institute, 2018). However, slow (linear) cooling from 48.9 to 26.7°C in 6 h per the 1999 guidelines could lead to the growth of *C. perfringens* greater than the 1-log limit, depending on product formulation (ComBase, 2023).

In 2017, the compliance guidelines were revised for quicker cooling during the temperature zone that supports the most rapid growth (Phase 1: 48.9 to 26.7°C; 120 to 80°F) in no more than 1 h, followed by chilling from 26.7 to 12.8°C (80 to 55°F) (Phase 2) in 5 h (for total time of 6 h) and then continuous chilling until 4.4°C (40°F) (Phase 3) (U.S. Department of Agriculture Food Safety and Inspection Service, 2017). After feedback from industry and academia, the 2021 FSIS guidelines recognized the difficulty in chilling large mass, non-intact products thicker than 11.5 cm in diameter to 26.7°C within 1 h, and identified cooling of this product as a "scientific gap" that needed further research (U.S. Department of Agriculture Food Safety and Inspection Service, 2021).

One recommended strategy to address this vulnerability (that is, the potential for *C. perfringens* to grow to infectious levels during cooling if present in the raw batter at high levels) is to add antimicrobial agents that have been validated as effective against the pathogen under the cooling conditions. Several clean-label antimicrobial agents, such as commercially available buffered vinegar and cultured sugar-vinegar products, have been shown to inhibit the growth of *Listeria monocytogenes* in cured and uncured meats (Delgado-Pando et al., 2021; Golden et al., 2014; McDonnell et al., 2013; Shrestha et al., 2022; Sijtsema et al., 2014; Weyker et al., 2016). Similar efficacy has been shown against *C. perfringens*, but testing of formulations and cooling conditions representing commercial conditions for high-moisture uncured meat is limited (King et al., 2015; Li et al., 2012; Smith et al., 2018; Valenzuela-Martinez et al., 2010). These ingredients contain single or combinations of organic acids or other antimicrobial compounds, but composition is proprietary. Given the wide variation of activity among these ingredients, the aim of this study is to screen several vinegar-based clean-label antimicrobials (added at equal concentrations) for the inhibition of *C. perfringens* growth in a high-moisture uncured turkey product when Phase 1 (48.9 to 26.7°C) cooling is extended up to 5 h.

Materials and Methods

Meat preparation

Frozen turkey breast was thawed at 4.4°C and ground through a 4.76-mm plate. A brine was made by combining all nonmeat ingredients (1.22% modified food starch, 0.3% sodium tripolyphosphate, 1.5% salt; w/w on total batch size basis; 25 kg final weight per batch), mixing until dissolved in a deionized water/ice mixture, and then adding to ground turkey and mixing for 2 min (temperature of the meat mixture estimated to be <4°C). The batter was divided (approximately 3000 g per treatment), and antimicrobial ingredients were added for the following formulations: (1) Control (no antimicrobials), (2) 1% dry vinegar (DV), (3) 1% dry vinegar-cultured sugar (DV-CS), or (4) 1% dry vinegar-fruit-spice-extract (DV-FSE). The pH values of the batches were adjusted as needed to 6.6 or 6.2 using sodium bicarbonate or 0.5N hydrochloric acid. The target post inoculation moisture was 75%, and salt was targeted to be 1.5%. Each treatment was stored in vacuum-sealed, oxygen-impermeable bags 3 mil high barrier pouches (12.8 cm × 23 cm; oxygen transmission rate 50 to 70 cm^3/m^2, 24 h at 25°C, and 60% relative humidity; water transmission rate 6 to 7.5 g/m^2, 24 h at 25°C, and 90% relative humidity; UltraSource, Kansas City, MO, USA) at −20°C for future use. Frozen samples were thawed at 4°C for 48 h before inoculation. Different lots of turkey and nonmeat ingredients were used for each replication.

No sensory analysis was conducted on the treatments created in the laboratory. However, the concentrations used in this study were within the limits recommended by the manufacturer and presumed to have no substantial adverse effects on sensory properties.

Bacterial spore preparation and inoculation of raw meat

C. perfringens spores were prepared, and meat was inoculated as previously described (Kennedy et al., 2013). Briefly, *C. perfringens* spore crops of strains ATCC 12915, ATCC 12916, and ATCC 13124 were prepared individually and enumerated. The inoculum was prepared with approximately equal concentrations of each spore strain and then diluted in sterile deionized water to deliver approximately 2.5-log CFU/g of meat. Each raw batter was inoculated using a 1% (v/w) liquid inoculum and mixed for 3 min (KitchenAid stand mixer, Benton Harbor, MI, USA). After mixing, the inoculated meat was portioned into oxygen- and moisture-impermeable bags (25 ± 0.5 g/pouch), flattened for uniform thickness (ca. 3 mm), and vacuum sealed (Multivac AGW, Sepp Haggemuller KG, Wolfertschewenden, Germany). Prepared samples were hung onto removable incubator racks using binder clips and spread to ensure even heat distribution. Samples were stored at 4°C overnight before cooking. Additional uninoculated samples from each formulation were prepared as described previously to monitor background microflora and for proximate analysis.

Cooking, cooling, and sampling

Racks with samples were placed in programmable incubators (Freeze/Thaw Chamber Model 7901-25-2, Caron, Marietta, OH, USA) and cooked until the internal temperature reached 70°C (158°F, 1 h come-up time). Samples were held at 70°C for 10 min, then cooled according to one of the 4 following cooling profiles. Each cooling profile consisted of 3 phases, which were Phase 1: 48.9–26.7°C (120–80°F), Phase 2: 26.7–12.8°C (80–55°F), and Phase 3: 12.8–4.4°C (55–40°F). The length of Phase 1 varied and was either 2, 3, 4, or 5 h. Phases 2 and 3 were fixed at 5 h each. Changes in internal sample temperature were monitored in real time via thermocouple probes (traceable thermometer and type K probe, Thermo Fisher Scientific, Waltham, MA, USA) that were inserted into individually packaged meat samples using a rubber septum (Chembond round patches, Tru-Flate, Plews and Edelman, Dixon, IL, USA).

Triplicate samples from each treatment were removed from the incubator and tested at 5 sampling points: raw batter post inoculation, post cook after temperature reached 48.9°C, and at internal temperatures of 26.7, 12.8, and 4.4°C. Samples within the bag were manually broken diluted (25 g: 50 mL Butterfield's Phosphate Buffer), and homogenized using a stomacher (IUL Masticator 400, Neutec Group Inc., Barcelona, Spain). Serial dilutions (1:10 in 0.1% peptone water) were spread plated onto duplicate plates of tryptose-sulfite-cycloserine agar (TSC) (Oxoid Ltd., Basingstoke, UK) with a TSC overlay. Plates were incubated for 24 h at 35°C under anaerobic conditions. All black colonies (anaerobic sulfite-reducing Clostridia) were counted for determining populations of *C. perfringens*; no additional confirmation was performed. Colony counts were converted to log CFU/g with a minimum detection limit of 1.48-log CFU/g. Uninoculated samples were assayed after cook and at the end of testing for aerobic (Plate Count Agar, Difco, BD, Sparks, MD, USA) and anaerobic plate counts (Oxoid Ltd., Basingstoke, UK). Growth is defined as greater than a 1-log increase over the average inoculum level (National Advisory Committee on Microbiological Criteria for Foods, 2010).

Physiochemical analysis

Triplicate uninoculated samples for each treatment were analyzed for pH, water activity (a_w), NaCl, and moisture at 0-time (post cook). The pH of each sample was measured by homogenizing a representative 10 g meat sample into 90 mL deionized water, and pH was measured on the slurry (Orion Star A111 pH meter and Orion 8104 combination electrode, Thermo Fisher Scientific, Waltham, MA, USA). Water activity was measured using a water activity meter (AquaLab 4TE, Decagon, Pullman, WA, USA), NaCl content was determined by AgNO3 potentiometric titration as % Cl- (model DL22 food and beverage analyzer, Mettler, Columbus, OH, USA), and moisture content was measured using the 5-h vacuum oven method OAC 950.46 (AOAC, 2000).

Statistical analysis

The experiment had 2 levels of pH, 4 antimicrobial treatments, and 4 Phase 1 cooling times, resulting in 32 runs that were replicated 2 times at 4 sampling intervals with 3 samples each for a total of 768 samples. Populations of *C. perfringens* were transformed into log values for analysis. Log change was calculated by subtracting the populations of each individual sample from the average pre-cook sampling point.

Data reported in Figures 1–4 for each treatment (formulation/cooling profile/pathogen) are the mean log change and standard deviations for both trials ($n = 6$) at the last sampling interval (end of total cooling), i.e., the greatest log change observed throughout the testing. LSMeans were calculated using JMP version 17.0.0 (JMP Statistical Discovery, LLC 2022). Means were separated using Tukey's Honest Significant Difference (HSD) levels sharing the same letter are not significantly different ($\alpha = 0.05$).

Results

Proximate analysis

Treatments tested at 0-time post cook met the targets for all parameters tested for both trials (Table 1). The average moisture across all samples for the 8 treatments was 74.85% ± 0.43%, and the average salt was 1.52% ± 0.05%. The average pH values were 6.23 ± 0.03 and 6.59 ± 0.04 for the 2 pH levels of 6.2 and 6.6, respectively. Water activity values were similar for all samples, 0.9820 ± 0.002.

C. perfringens growth and inhibition

As expected, uncured, high-moisture turkey product without antimicrobials supported greater than a 1-log increase of *C. perfringens* when Phase 1 cooling from 48.9 to 26.7°C was extended beyond the recommended 1-h limit (Figures 1–4) (U.S. Department of Agriculture Food Safety and Inspection Service, 2021). For products with 75% moisture, 1.5% NaCl, and pH 6.6, populations of the pathogen increased by 1.94 ± 0.21, 3.51 ± 0.44, 5.33 ± 0.42, and 6.11 ± 0.54 log when Phase 1 cooling was extended to 2, 3, 4, and 5 h, respectively. The average growth for products formulated with pH 6.2 was slightly lower, with increases of 1.73 ± 0.26, 2.40 ± 0.62, 4.84 ± 0.08, and 6.09 ± 1.23 log for the 4 cooling profiles, respectively, but the differences for growth between the pH values were not significant ($P > 0.05$).

In contrast, the addition of any of the 3 DV-based ingredients to the turkey product inhibited the growth of *C. perfringens* (<0.5-log difference from inoculated levels) at both pH 6.2 and 6.6 when Phase 1 cooling was extended to 2 h (12 h total cooling, Figure 1). When Phase 1 cooling was extended to 3 h, inhibition by the 3 antimicrobials was similar in products with pH 6.2 (Figure 2). However, greater differences were observed among the 3 ingredients in pH 6.6 products (Figure 2). *C. perfringens* grew by 1.26 ± 0.21 and 1.20 ± 0.30 log in the DV and DV-FSE treatments, respectively, at pH 6.6, but only 0.40 ± 0.17 and 0.53 ± 0.10 log difference (within plating error), respectively, at pH 6.2 treatments. No growth was observed in the DV-CS treatments at either pH.

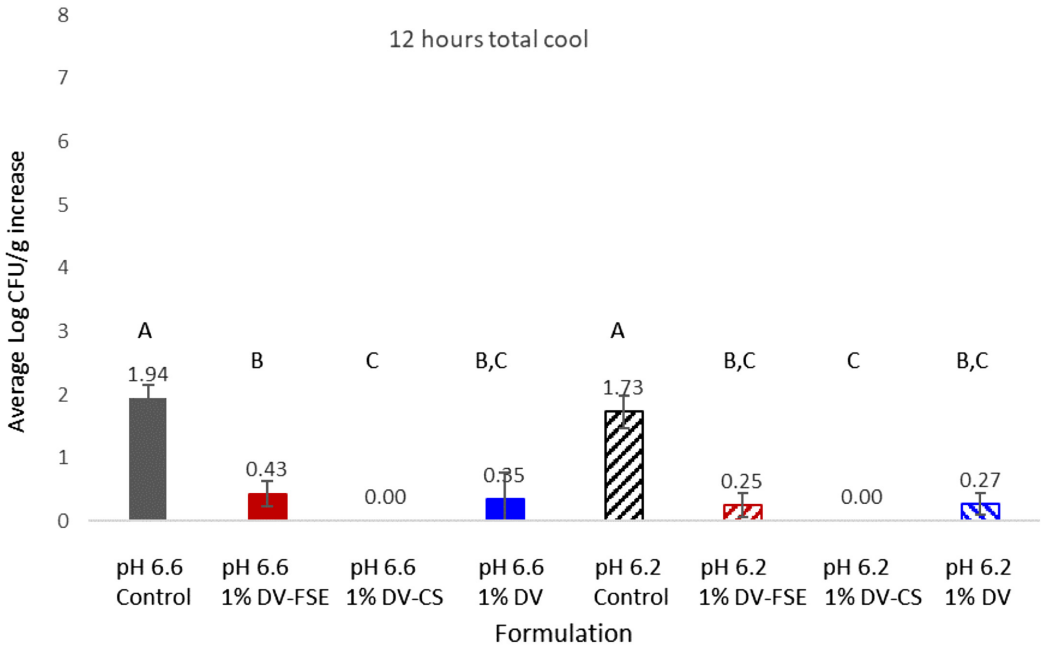

Figure 1. Changes in populations of *Clostridium perfringens* (log CFU/g) in uncured turkey product with extended Phase 1 cooling from 48.9 to 26.7°C (120–80°F) of 2 h, Phase 2 from 26.7 to 12.8°C (80–55°F) of 5 h, and Phase 3 from 12.8 to 4.4°C (55–40°F) of 5 h. Data reported from the end of total cooling (largest log change). Levels sharing the same letter are not significantly different.

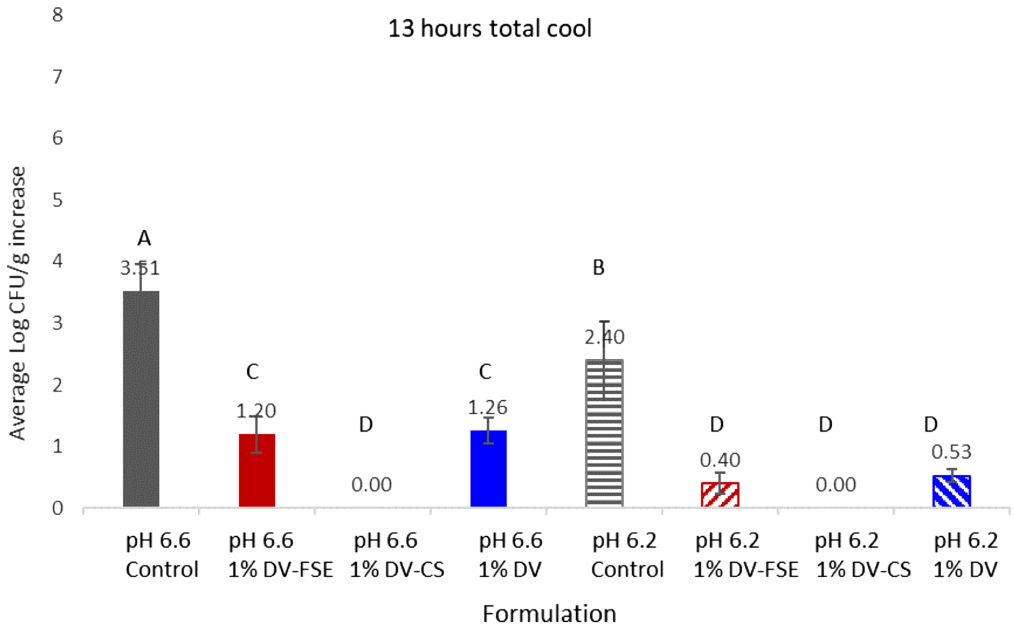

Figure 2. Changes in populations of *Clostridium perfringens* in uncured turkey product with extended Phase 1 cooling from 48.9 to 26.7°C (120–80°F) of 3 h, Phase 2 from 26.7 to 12.8°C (80–55°F) of 5 h, and Phase 3 from 12.8 to 4.4°C (55–40°F) of 5 h. Data reported from the end of total cooling (largest log change). Levels sharing the same letter are not significantly different.

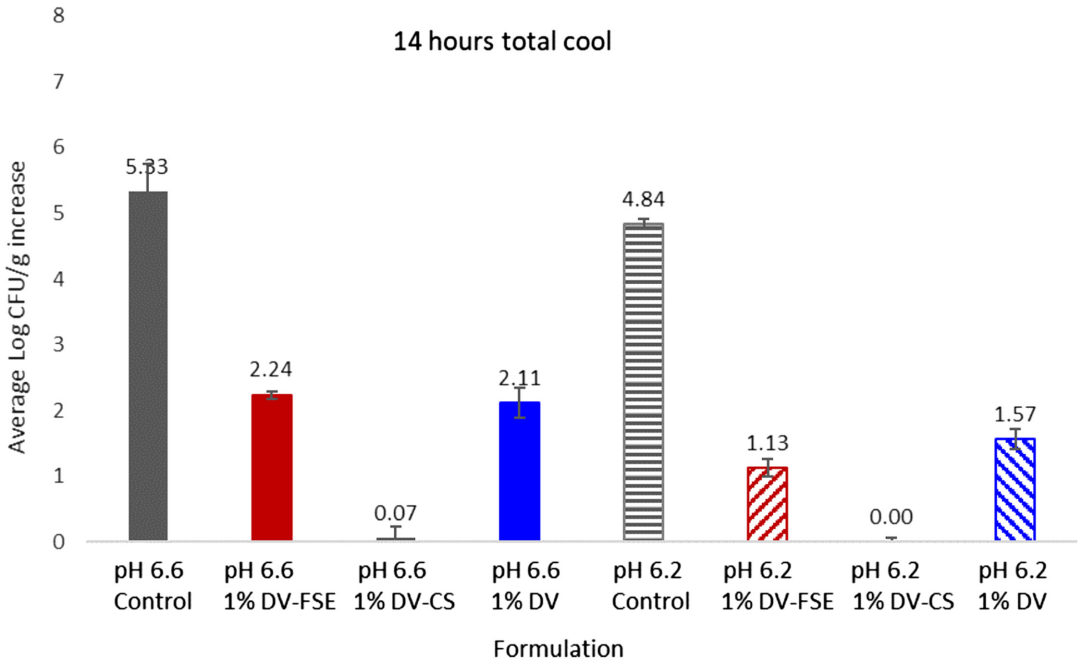

Figure 3. Changes in populations of *Clostridium perfringens* in uncured turkey product with extended Phase 1 cooling from 48.9 to 26.7°C (120–80°F) of 4 h, Phase 2 from 26.7 to 12.8°C (80–55°F) of 5 h, and Phase 3 from 12.8 to 4.4°C (55–40°F) of 5 h. Data reported from the end of total cooling (largest log change). Levels sharing the same letter are not significantly different.

Of the 3 DV-based antimicrobials, the DV-CS treatments demonstrated significantly greater inhibition ($P < 0.05$) than the other 2 ingredients when Phase 1 cooling was extended to both 4 and 5 h (Figures 3 and 4). No growth (<0.5-log difference from initial inoculum) was observed for the 4-h Phase 1 cooling in either pH 6.2 and 6.6 treatments formulated with the 1% DV-CS, compared to 2.11 ± 0.23 and 1.57 ± 0.16 for DV treatments with pH 6.6 and 6.2, respectively, and 2.24 ± 0.06 and 1.13 ± 0.14 log

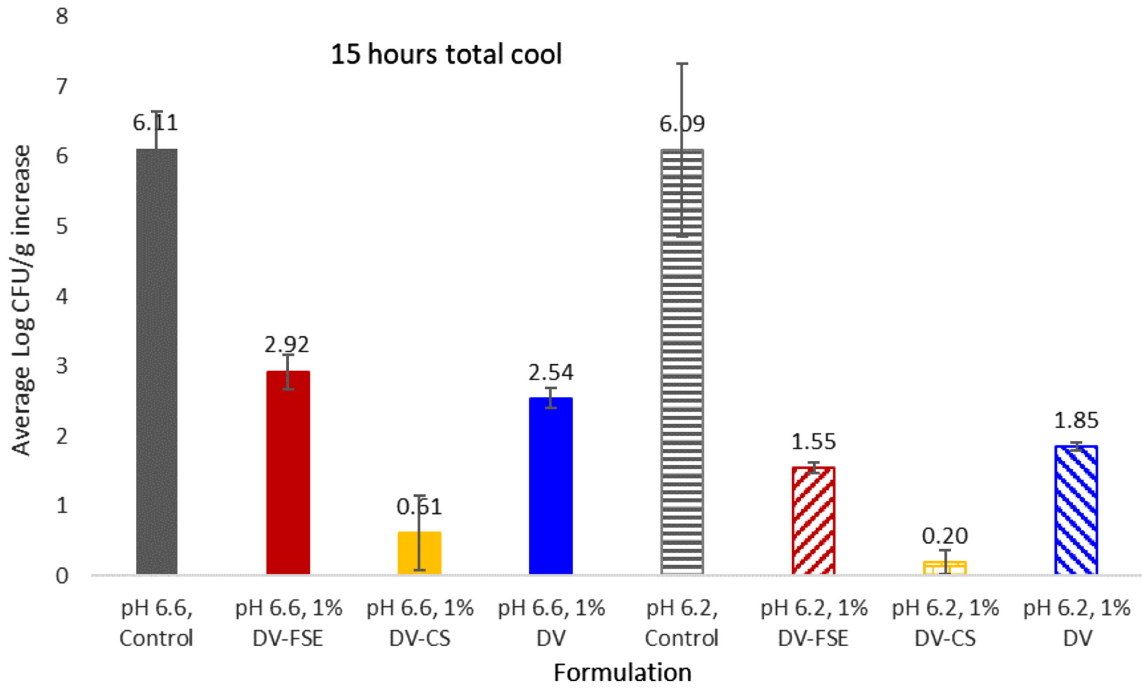

Figure 4. Changes in populations of *Clostridium perfringens* in uncured turkey product with extended Phase 1 cooling from 48.9 to 26.7°C (120–80°F) of 5 h, Phase 2 from 26.7 to 12.8°C (80–55°F) of 5 h, and Phase 3 from 12.8 to 4.4°C (55–40°F) of 5 h. Data reported from the end of total cooling (largest log change). Levels sharing the same letter are not significantly different.

Table 1. Physiochemical analysis average and standard deviation for duplicate trials ($n = 6$) of 8 model uncured turkey products supplemented with 1% dry vinegar-based clean-label antimicrobials.

Treatment—Antimicrobial, Target pH	% Moisture	% Salt	pH	a_w
Treatment 1—Control, 6.6	74.58 ± 0.44	1.49 ± 0.02	6.58 ± 0.02	0.985 ± 0.00
Treatment 2—Dry vinegar, 6.6	75.16 ± 0.31	1.48 ± 0.02	6.57 ± 0.02	0.980 ± 0.00
Treatment 3—Dry vinegar-cultured sugar 6.6	75.00 ± 0.26	1.52 ± 0.03	6.61 ± 0.04	0.981 ± 0.00
Treatment 4—Dry vinegar–fruit-spice extract, 6.6	75.40 ± 0.26	1.58 ± 0.02	6.62 ± 0.05	0.980 ± 0.00
Treatment 5—Control, 6.2	74.44 ± 0.17	1.51 ± 0.07	6.24 ± 0.03	0.985 ± 0.00
Treatment 6—Dry vinegar, 6.2	74.79 ± 0.26	1.49 ± 0.04	6.22 ± 0.03	0.981 ± 0.00
Treatment 7—Dry vinegar-cultured sugar, 6.2	74.51 ± 0.29	1.58 ± 0.02	6.21 ± 0.02	0.982 ± 0.00
Treatment 8—Dry vinegar–fruit-spice extract, 6.2	75.00 ± 0.19	1.55 ± 0.04	6.25 ± 0.04	0.980 ± 0.00

increase for the 2 DV-FSE treatments, respectively (Figure 3). When Phase 1 cooling was extended to 5 h (Figure 4), populations increased by 2.54 ± 0.15, 2.92 ± 0.25, and 0.61 ± 0.53 for the DV, DV-FSE, and DV-CS treatments at pH 6.6, respectively. At pH 6.2, populations increased 1.85 ± 0.06, 1.55 ± 0.07, and 0.20 ± 0.17 for the 3 treatments, respectively.

Discussion

This study demonstrated the efficacy of vinegar-based clean-label antimicrobial ingredients, alone or blended with other ingredients, to inhibit the growth of *C. perfringens* in high-moisture uncured poultry products. King et al. previously demonstrated that 1% fruit-spice extract (no vinegar) and 1% culture sugar-blend inhibited *C. perfringens* growth in uncured turkey (ca. 75% moisture, 1.2% NaCl, pH 6.3) when products were cooled from 54.4 to 26.7°C in 5 h, but supplementing product with 0.7% DV allowed over a 2-log increase under the same conditions (King et al., 2015). Other studies reported consistent inhibition of *C. perfringens* by buffered vinegar and lemon juice concentrate in uncured turkey and roast beef (Li et al., 2012), but the pH values tested in those reports (5.6 to 5.8) could further decrease growth compared to the pH values for the current study (6.2 to 6.6) (ComBase, 2023).

Plant extracts can contain a mixture of antimicrobial compounds such as organic acids, terpenes, and

polyphenols, but levels of activity will depend on the source of the extract and potentially even the cultivar (López-Malo et al., 2020; Suriyaprom et al., 2022). Similarly, fermentates (such as cultured sugar) will be a source of one or more organic acids (frequently lactic or propionic acid), but can also contain bacteriocins or other antimicrobial peptides (Elsser-Gravesen and Elsser-Gravesen, 2014). The type and level of active compound(s) will depend on the culture used, substrate, culture conditions, and blending/standardization during production.

Organic acids are more effective in their undissociated form; hence, the order of efficacy in a food frequently follows their respective dissociation constant (pK_a), the pH at which 50% of the total acid is dissociated. Compounds having higher pK_a values (such as acetic with pK_a 4.75) typically have greater efficacy than acids with lower pK_a (such as lactic acid with acid pK_a 3.79) (Davidson et al., 2005). Therefore, at any given pH and using only a single acid, the DV compounds would be expected to have greater antimicrobial activity than lactic acid alone. However, previous studies have shown that sodium lactate alone was as effective as similar concentrations of lactate–diacetate blend (Juneja and Thippareddi, 2004). This suggests that the mechanism of action of the cultured sugar-vinegar products against *Clostridium* species is more complex than the effect of dissociated acid. An alternative proposed mechanism for the action of lactate against *Clostridium botulinum* was the inhibition of a major anaerobic energy metabolism pathway necessary for growth (Maas et al., 1989).

Efficacy as an antimicrobial will depend on not only the composition of the fermentate but also the target microorganism. Propionic acid-based ingredients are more effective against *L. monocytogenes* than lactic acid-based fermentates when used at levels that will not affect sensory properties (Engstrom et al., 2021; Engstrom et al., 2020), but data suggest that they are less effective against *C. perfringens* (Glass et al., 2024). In both cases of plant extracts and fermentates, commercially available products are proprietary, and the type and level of active components are typically not disclosed on a product data sheet. Vinegar (either dry or liquid-buffered vinegar) is a known source of acetic acid; the concentration of acetic acid is typically similar among various sources for DV but may vary for liquid vinegars.

This study confirms previous findings that cooling rate, formulation (pH, moisture, salt), and antimicrobials are critical factors in the control of *C. perfringens* in cured and uncured meat and poultry products (Glass et al., 2024; ComBase, 2023; Kennedy et al., 2013; King et al., 2015; Osterbauer et al., 2017; U.S. Department of Agriculture Food Safety and Inspection Service, 2021). For uncured poultry products, the addition of 1% DV delays growth when FSIS Option 2 Phase 1 cooling (48.9 to 26.7°C) is extended beyond the 1-h limit to ≥2 h. Blends of DV with cultured sugars or fruit-spice extract will further delay growth, but due to the variability of activity among wide portfolios of available products, validation studies for individual ingredients are still needed to confirm efficacy.

Acknowledgments

We extend our gratitude to the team members of the Applied Food Safety Laboratory at the University of Wisconsin-Madison Food Research Institute (FRI), including Brandon Wanless, Quinn Singer, Jie Yin Lim, and Christie Cheng for their technical assistance. This research was funded by the University of Wisconsin-Madison FRI Undergraduate Summer Scholar Program and by unrestricted gifts to the FRI from the food industry.

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