Reducing spoilage in intermediate moisture pet foods using food-safe additives as a model system

by

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B.S., Tamilnadu Agricultural University, 2009
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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Grain Science & Industry
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Manhattan, Kansas

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Abstract

Safety of foods, including pet food, is affected by the presence of pathogenic bacteria, molds, and mite infestation. Intermediate moisture foods (IMFs) with water activity (a_w) of 0.60-0.85 are susceptible to storage mite and mold contamination. Control of mites depends on chemical methods such as fumigation. Methyl bromide which was used as a fumigant for stored products is now banned in many countries because of its ozone-depleting effects. Effective alternatives to methyl bromide are needed to develop integrated pest management programs for mites. Another risk for human and animal food safety is spoilage and mycotoxin contamination due to the presence of storage molds. Synthetic mold inhibitors like potassium sorbate have been used to control mold growth in IMFs but a natural alternative is desired for 'clean labeling' of foods in accordance with the current consumer trend. Liquid smoke is a naturally derived flavor and preservative with known antimicrobial properties; it may have potential antimycotic and acaricidal properties. Even though IMFs are below the minimum water activity for most bacterial growth (0.90 a_w) they are susceptible to pathogenic bacteria through post processing cross-contamination. The organic acid 2-hydroxy-4-methylthio-butanoic acid (HMTBa) has been used as a methionine supplement in animal feed and potentially has antimicrobial effects. The first objective of this study was to investigate the efficacy of eight liquid smoke preparations on the survivorship and orientation behavior of the cheese or mold mite, *Tyrophagus putrescentiae* Schrank (Acaridae: Sarcoptiformes), in semi-moist pet food. Survival of *T. putrescentiae* on treated (0.3% smoke inclusion) and untreated (0% smoke) pet food samples was determined by enumerating their population at 7, 14, and 28 days post-infestation. Further, semi-moist pet food cubes were dipped in smoke preparations at 0, 0.3, 1, 5, 10, 25, 50, and 100% concentration, and used in two-choice behavioral assays. The attraction or repulsion of mites towards treatments was determined by the

repellency index (RI). The second objective of this study was to determine the effects of liquid smoke on shelf-life and growth of the storage mold, *Aspergillus flavus* Link (Trichocomaceae: Eurotiales), in semi-moist pet food with liquid smoke inclusion at 0, 0.5, 1, 2 and 4% (w/w). Shelf-life was estimated by storing samples at 28°C and 65-70% RH over 30 days and recording the number of days for the appearance of visible wild-type mold. In another experiment, samples inoculated with *A. flavus* were analyzed for fungal growth at sampling intervals of 2 days over a 35-day period. The third objective of this study was to evaluate the antimicrobial and residual effects of two types of organic acid mixtures, Activate DA™ and Activate WD-MAX™, containing HMTBa against *Salmonella enterica* Le Minor & Popoff (Enterobacteriaceae: Enterobacterales), *Escherichia coli* Castellani & Chalmers (Enterobacteriaceae: Enterobacterales), and *A. flavus* in pet food kibbles. Activate DA at 0, 1, and 2% (w/w), and Activate WD-MAX at 0, 0.5, and 1% (w/w) were tested as coating on the kibbles inoculated with the target pathogens. The fourth and final objective of this study was to evaluate Activate DA and WD-MAX on food contact surfaces like rubber, plastic, stainless steel, and concrete against *Salmonella enterica* contamination. Results from the studies with liquid smoke against mites indicated that the smoke did not kill or inhibit mite population growth when compared to the untreated; however, smoke preparations with high carbonyl content may provide repellency to retard mite infestation in semi-moist pet food. The shelf-life study indicated that smoke preparations with high carbonyl, and medium to low phenol content were the most effective in prolonging the number of days to occurrence of visible mold (26-28 days) compared to the untreated (7.7 days). The mold challenge study with *A. flavus* indicated that smoke preparations containing high carbonyls and low phenols significantly reduced mold counts by 1, 1.7, and 2.5 logs when compared to the untreated at 1, 2, and 4% concentrations, respectively. Studies with the organic acid mixtures coating on pet food

kibbles indicated that Activate DA at 2% and Activate WD-MAX at 1% decreased *Salmonella* counts by 4 to 4.6 logs, and *E. coli* counts by ~3 logs after 24 h compared to the untreated samples. Levels of *A. flavus* did not vary up to 7 days, and subsequently started to decline by ~3.8 logs after 28 days for Activate DA and WD-MAX at 1 and 2%, respectively. At 1%, Activate WD-MAX reduced *Salmonella* counts by 3.1 logs on stainless steel surfaces, which was the highest, and 0.2 logs on rubber from automobile tire which was the lowest, across treatments. On both kibbles and surfaces Activate WD-MAX was more effective at a lower concentration (1%) than Activate DA (2%). Evaluating residual effect of the organic acid mixtures indicated that across treatment levels, Activate DA and WD-MAX had similar effect in reducing *Salmonella* counts by 4.2-4.4 logs on day 30, and 3.4-3.6 logs on day 90, compared to 4.3-4.4 logs on day 1. Across days, there was difference in the residual efficacy of organic acid mixtures on day 90, which was lesser by 0.8 logs, compared to day 1 and day 30.

Key words: Semi moist pet food, Liquid smoke, HMTBa, *Tyrophagus putrescentiae, Aspergillus flavus, Salmonella*

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Key words: Semi moist pet food, Kibble, Liquid smoke, HMTBa, *Tyrophagus putrescentiae*, *Aspergillus flavus, Salmonella*

Table of Contents

List of Tables v
List of Figures viii
Acknowledgements xx
Dedication xxi
Chapter 1 - Literature review 1
Introduction 1
Food safety issues in semi-moist and low moisture foods 3
Mold contamination 6
Molds and mycotoxins affecting semi-moist foods 6
Mold inhibitors commonly used in the food industry 10
Mode of action of natural mold inhibitors 13
Mite infestation 14
Mite infestation in stored food products 14
Methods to control mite infestation 16
Contamination of food contact surfaces 18
Contaminants on surfaces in the food industry 18
Sanitizers used to decontaminate surfaces 19
Rationale for this research 21
Liquid smoke 23
Application of liquid smoke in the food industry 23
Generation of liquid smoke 24
Composition of liquid smoke 25
Antimicrobial activity of liquid smoke 27
Organic acid mixtures – Activate DA™ and Activate WD-MAX™ 31
Uses of HMTBa as a feed supplement and an antimicrobial 32
Conclusion 33
Hypotheses 34
Objectives 34
References 36

Chapter 2 - Efficacy of liquid smoke preparations to mitigate infestation of the storage mite, *Tyrophagus putrescentiae*, in a model semi-moist pet food system 66
Abstract 67
Introduction 69
Materials and Methods 72
Preparation of semi-moist pet food and source of liquid smoke 72
Mite culture 72
Treatments used in the study 73
Experiment 1: Mite population growth assay 73
Experiment 2: Mite orientation behavior assay 74
Statistical analysis 75
Results 76
Discussion 77
Conclusion 84
References 85
Tables & Figures 94
Chapter 3 - Effects of liquid smoke preparations on shelf life and growth of wild-type mold and *Aspergillus flavus* in a model semi-moist pet food system 103
Abstract 104
Introduction 106
Materials and Methods 108
Fungal cultures 108
Preparation of semi-moist pet food and source of liquid smoke 109
Experiment 1: Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) 110
Experiment 2: Days-to-mold (shelf life) study 111
Experiment 3: Mold challenge study with *Aspergillus flavus* 111
Statistical analysis 112
Results 112
Discussion 115
Conclusion 121

References 122
Tables & Figures 127
Chapter 4 - Use of organic acid mixtures to mitigate *Salmonella enterica, Escherichia coli* and *Aspergillus flavus* on dry pet food kibbles 139
Abstract 140
Introduction 142
Materials & Methods 147
Manufacturing of dry pet food kibbles 147
Sources of organic acid mixtures 147
Preparation of *Salmonella, Escherichia coli*, and *Aspergillus flavus* inoculum 147
Experiment 1: Minimum inhibitory (MIC), bactericidal (MBC) and fungicidal concentrations (MFC) 148
Experiment 2: *Salmonella, Escherichia coli* and *Aspergillus flavus* mitigation on dry dog food kibbles coated with organic acid mixtures 149
Experiment 3: Residual antimicrobial effect of organic acid mixtures in dry pet food kibbles over time to mitigate exposure to *Salmonella* 151
Statistical analysis 152
Results 153
Discussion 157
Conclusion 163
References 164
Chapter 5 - Use of organic acid mixtures to mitigate *Salmonella* on food contact surfaces 185
Abstract 186
Introduction 188
Materials & Methods 191
Sources of organic acid mixtures 191
Preparation of *Salmonella* cocktail inoculum 191
Contact surfaces and *Salmonella* inoculation 192
Confirmative test for *Salmonella* 193
Statistical analysis 194
Results 194

Discussion 196
Conclusion 201
References 203
Tables & Figures 210

List of Tables

Table 1.1. Microorganisms growing in the a_w range of intermediate moisture foods……..…3

Table 1.2. Minimum water activity limits for the growth of common mold species found in intermediate moisture foods…….6

Table 1.3. Natural/clean label ingredients evaluated to inhibit mold growth in foods……..11

Table 1.4. Natural/GRAS (generally recognized as safe) ingredients evaluated in food substances to mitigate *T. putrescentiae* infestation……..16

Table 1.5. Compounds primarily identified in wood smoke…….26

Table 1.6. Liquid smoke evaluated as an antimicrobial in foods……..27

Table 2.1. Formula used for manufacturing the model semi-moist pet food…….93

Table 2.2. Liquid smoke preparations evaluated in the study……94

Table 2.3 Mean mite population growth at 7, and 14 days on semi-moist pet food treatments with inclusion of liquid smoke preparations namely P-1720, Cloud S-5 and Cloud S-C100 at 0.3% w/w, in comparison to the untreated control (semi-moist pet food with no smoke added), and the mite rearing diet (lab culture diet). Propylene glycol solution treatment (positive control) did not show mite population growth……95

Table 2.4 Mean mite repellency indices for the liquid smoke preparations at 100%, 50%, 25%, 10%, 5%, 1% and 0.3%, coated on semi-moist pet food at enumeration time points 1 h, 8 h, and 24 h. Semi-moist pet food coated with distilled water served as the untreated control and 20% propylene glycol treatment served as the positive control. Positive control showed 100% repellency. RI values which are "+" indicate repellency and "-" indicate attraction……...96

Table 3.1. Formula used for manufacturing the model semi-moist pet food……...124

Table 3.2. Liquid smoke preparations evaluated in the study……..125

Table 3.3. Water activity (a_w) and pH of semi-moist pet food treatments without and with inclusion of liquid smoke (0.5% - 4%, w/w)……...126

Table 3.4. Minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) of liquid smoke preparations (S1 to S8) in nutrient broth (PDB) against *Aspergillus flavus* and *Fusarium graminearum* in comparison with potassium sorbate, a known mold inhibitor. A positive control consisted of fungal inoculum only (no

treatment), and a negative control consisted of potato dextrose broth (PDB) alone (no treatment, no fungal inoculation) (not shown)…….127
Table 3.5. Mean number of days taken to observe wild-type mold growth in a model semi-moist pet food with inclusion of liquid smoke treatments (S1 to S8) at 0.5%, 1%, 2% and 4% in comparison with untreated (0% smoke). Potassium sorbate treatment (positive control) did not show visible mold growth during the 30-day period (not shown)……128
Table 3.6. Mean logarithmic reduction (between day 0 and day 35) of *Aspergillus flavus* counts in semi-moist pet food treatments with inclusion of liquid smoke preparations (S1 to S8) at 0.5%, 1%, 2% and 4% concentrations in comparison with untreated (0% smoke) over 35 days incubation period. Initial load of *A. flavus* inoculated in the semi-moist pet food samples was 3.5 log CFU/mL. Potassium sorbate treatment (positive control) did not show mold growth during the 35-day period (not shown)……129
Table 3.7. Linear regression (linear model $y = a + bx$) parameters of logarithmic reduction of *Aspergillus flavus* counts in semi-moist pet food treatments with inclusion of liquid smoke preparations (S1 to S8) at 2% and 4% concentrations……130
Table 4.1. Formulation of uncoated dry dog food kibbles……..169
Table 4.2. Minimum inhibitory concentrations (MICs), minimum bactericidal (MBCs) and fungicidal concentrations (MFCs) of organic acid mixtures Activate DA and Activate WD-MAX in nutrient broth (TSB or PDB) against *Salmonella, E. coli* (STEC), and *Aspergillus flavus*…….170
Table 4.3. Mean logarithmic reduction of *Salmonella* counts in pet food kibble treatments with inclusion of organic acid mixtures Activate DA at 1% and 2% and Activate WD-MAX at 0.5% and 1% concentrations as coating, in comparison with untreated (0% organic acid). A negative control consisted of canola oil coated kibble (no organic acid, no inoculation)……..……171
Table 4.4. Mean logarithmic reduction of *E. coli* counts in pet food kibble treatments with inclusion of organic acid mixtures Activate DA at 1% and 2% and Activate WD-MAX at 0.5% and 1% concentrations as coating, in comparison with untreated (0% organic acid). A negative control consisted of canola oil coated kibble (no organic acid, no inoculation)……..172

Table 4.5. Mean logarithmic reduction of *Aspergillus flavus* counts in pet food kibble treatments with inclusion of organic acid mixtures Activate DA at 1% and 2% and Activate WD-MAX at 0.5% and 1% concentrations as coating, in comparison with untreated (0% organic acid). A negative control consisted of canola oil coated kibble (no organic acid, no inoculation) (not shown)……..……...173

Table 4.6. Linear regression (linear model $y = a + bx$) parameters of logarithmic reduction of *Salmonella* and *E. coli* counts in pet food kibble treatments with inclusion of organic acid mixtures Activate DA at 1% and 2% and Activate WD-MAX at 0.5% and 1% concentrations as coating…….……174

Table 4.7. Mean logarithmic reduction of *Salmonella* counts in pet food kibble treatments with inclusion of organic acid mixtures Activate DA at 1% and 2% and Activate WD-MAX at 0.5% and 1% concentrations as coating, in comparison with untreated (0% organic acid) showing residual effect of treatments over 1-, 30-, and 90-days during storage. A negative control consisted of canola oil coated kibble (no organic acid, no inoculation) (not shown)……..……175

Table 5.1. Mean logarithmic reduction (log CFU/cm^2) of *Salmonella* counts in pet food contact surfaces treated with organic acid mixtures Activate DA at 1% and 2% and Activate WD-MAX at 0.5% and 1% concentrations in comparison with untreated (0% organic acid). Formaldehyde 30% treatment (positive control) had complete kill of *Salmonella* counts in 24 h (not shown) …….……205

List of Figures

Fig 2.1. Bioassay arena used in two-choice behavioral test for mite orientation study…….97

Fig. 2.2. Mean mite population growth at 7, and 14 days on semi-moist pet food treatments with inclusion of liquid smoke preparations namely P-1720, Cloud S-5 and Cloud S-C100 at 0.3% w/w, in comparison to the untreated control (semi-moist pet food with no smoke added), the standard mite rearing diet (lab culture diet), and 20% propylene glycol solution (positive control)……..98

Fig. 2.3. Mean mite repellency indices (values which are '+' indicate repellency and '-' indicate attraction) for the liquid smoke preparations namely P-1720, Cloud S-5, Cloud S-C100, Black deli, Hickory OS-1473, Code 10, Code V and Cloud S-AC15 at concentrations 100%, 50%, 25%, 10%, 5%, 1% and 0.3%, coated on semi-moist pet food at enumeration time points 1 h, 8 h, and 24 h. Semi-moist pet food coated with distilled water served as the untreated control and 20% propylene glycol treatment served as the positive control……99

Fig. 3.1. Minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) of liquid smoke preparations (S1 to S8) in nutrient broth (PDB) against *Aspergillus flavus* in comparison with potassium sorbate, a known mold inhibitor. A positive control consisted of fungal inoculum only (no treatment), and a negative control consisted of potato dextrose broth (PDB) alone (no treatment, no fungal inoculation) (not shown)……..……131

Fig. 3.2. Minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) of liquid smoke preparations (S1 to S8) in nutrient broth (PDB) against *Fusarium graminearum* in comparison to potassium sorbate, a known mold inhibitor. A positive control consisted of fungal inoculum only (no treatment), and a negative control consisted of potato dextrose broth (PDB) alone (no treatment, no fungal inoculation) (not shown)……...……...132

Fig. 3.3. Mean number of days taken to observe wild-type mold growth in a model semi-moist pet food with inclusion of liquid smoke treatments (S1 to S8) at 0.5%, 1%, 2% and 4% in comparison with untreated (0% smoke). Potassium sorbate treatment (positive control) did not show visible mold growth during the 30-day period……...133

Fig. 3.4. Mean logarithmic counts (log CFU/mL) of *Aspergillus flavus* in semi-moist pet food treatments with inclusion of liquid smoke preparations (S1 to S8) at 0.5%, 1%, 2% and 4% concentrations in comparison with untreated (0% smoke). Potassium sorbate treatment (PC; positive control) did not show mold growth during the 35-day period. The detection limit is 1.0 log CFU/mL for sampling using PDA plates…….134

Fig 4.1. Comparison of chemical structures of HMTBa (methionine hydroxy analogue) vs. Methionine (amino acid)……..176

Fig. 4.2. Mean logarithmic counts (log CFU/mL) of *Salmonella* in pet food kibble treatments with inclusion of organic acid mixtures Activate DA at 1% and 2% and Activate WD-MAX at 0.5% and 1% concentrations as coating in comparison with untreated (0% organic acid). 0 h denotes initial load of *Salmonella* in inoculum before inoculation to the kibble. A negative control consisted of canola oil coated kibble (no organic acid, no inoculation). Limit of detection is 1 log CFU/mL for this study……...…..177

Fig. 4.3. Mean logarithmic counts (log CFU/mL) of *E. coli* in pet food kibble treatments with inclusion of organic acid mixtures Activate DA at 1% and 2% and Activate WD-MAX at 0.5% and 1% concentrations as coating in comparison with untreated (0% organic acid). 0 h denotes initial load of *E. coli* in inoculum before inoculation to the kibble. A negative control consisted of canola oil coated kibble (no organic acid, no inoculation). Limit of detection is 1 log CFU/mL for this study…….....178

Fig. 4.4. Mean logarithmic counts (log CFU/mL) of *E. coli* in pet food kibble treatments with inclusion of organic acid mixtures Activate DA at 1% and 2% and Activate WD-MAX at 0.5% and 1% concentrations as coating in comparison with untreated (0% organic acid). 0 h denotes initial load of *E. coli* in inoculum before inoculation to the kibble. A negative control consisted of canola oil coated kibble (no organic acid, no inoculation). Limit of detection is 1 log CFU/mL for this study…….....179

Fig. 4.5. Mean logarithmic counts of *Salmonella* in pet food kibble treatments with inclusion of organic acid mixtures Activate DA at 1% and 2% and Activate WD-MAX at 0.5% and 1% concentrations as coating, in comparison with untreated (0% organic acid) to investigate residual effect of treatments over 1-, 30- and 90-days after exposure to *Salmonella*. A negative control consisted of canola oil coated kibble (no organic acid, no inoculation). Limit of detection is 1 log CFU/mL for this study…….180

Fig. 5.1. Mean logarithmic counts (log CFU/cm^2) of *Salmonella* on food contact surfaces treated with organic acid mixture Activate DA at 1% and 2% concentrations in comparison with untreated (0% organic acid) and positive control (30% formaldehyde). Limit of detection is 1 log CFU/mL for this study……..…..206

Fig. 5.2. Mean logarithmic counts (log CFU/cm^2) of *Salmonella* in food contact surfaces treated with organic acid mixture Activate WD-MAX at 0.5% and 1% concentrations in comparison with untreated (0% organic acid) and positive control (30% formaldehyde). Limit of detection is 1 log CFU/mL for this study…….…….207

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Dedication

I humbly dedicate this work to the Almighty, my loving family, and respected teachers whose blessings, encouragement, and prayers have always helped me scale greater heights in life.

Chapter 1 - Literature review

Introduction

Semi-moist foods or intermediate moisture foods have gained more attention worldwide having features very similar to fresh food products, but with a longer shelf life (Qiu et al., 2019). By definition, intermediate moisture foods are shelf-stable food products that have water activities (a_w) of 0.60-0.84, with a moisture content ranging from 15-40% and are edible without rehydration (US-FDA, 2016). Historically, ancient civilizations produced intermediate moisture foods using methods such as sun drying, smoking or roasting over fire, and adding salt to preserve food for winter months (Barbosa-Cánovas et al., 2020). In the current times, foods are processed to intermediate moisture levels mostly by partial drying (e.g., dried fruit), osmotic drying using humectants (e.g., pickled vegetables), and food formulations using humectants and additives to keep them shelf stable (e.g., cakes, chewy dog treats). Many types of foods that are commercially available, like confectionery and pet foods, are specially formulated to achieve water activity in the semi-moist or intermediate moisture range (0.60-0.84 a_w). Food ingredients are mixed with salt and/or sugar, and additives such as polyols (e.g., propylene glycol) and mold inhibitors (e.g., potassium sorbate), and subjected to processing methods such as cooking, extrusion, baking or dehydration to result in an intermediate moisture final product (Fellows, 2009). This method of formulating foods is fast and energy efficient and offers great flexibility in formulation.

Production of semi-moist foods with properties close to fresh foods yet having extended shelf life to satisfy the demand of the consumer is vitally important for the food industry. The unique features that make semi-moist foods appeal to consumers include microbial safety, desirable odors, high nutritional values, and ready to eat (RTE) characteristics (Severini et al., 2008). Some of the popular examples of intermediate moisture foods consumed in our day-to-day

lives are fruit cake, country-style ham, fondants, high sugar cakes, sweetened condensed milk, salted fish, molasses, jams, pet food, dried fruits and nuts, parmesan cheese, chocolate bars, marshmallows, and biscuits. As these semi-moist food products encompass a wide category of foods ranging from cereal-based to meat-based to dairy products to pet foods, research in this area of foods contributes tremendously to the well-being of humans all over the world.

Semi-moist and low moisture pet foods (e.g., chewy dog treats and dog kibbles, respectively) are some of the most recent and modern formulated foods in the intermediate moisture and low moisture food categories. Semi-moist pet foods are a smaller but significant portion of the manufactured pet food market, and they require the use of humectants and acidification to control water activity and mold growth. Semi-moist pet foods also have a low fiber content and relatively high sugar content, which make them highly palatable for pets and increasingly purchased by pet owners (Zicker, 2008). Semi-moist pet foods are manufactured in a way similar to extruded food, but the water content is maintained at a higher level because of the added humectants. The final moisture content of 25 to 35% is more prone to mold growth and spoilage, which is mitigated by mold or bacterial inhibitors as well as managing the ‘active water’ component of the food (Zicker, 2008). The amount of water activity is a measure of the amount of water available for bacterial growth and the addition of humectants helps to keep this at a low level, which effectively inhibits their growth despite a higher total water content (Zicker, 2008). It is apparent that much effort is put toward producing products that not only meet nutrient targets but that are also safe for their intended purposes. In addition to the care paid to details during the formulation and manufacturing process, companies maintain post manufacturing quality controls that further ensure safety and nutritional adequacy for the products. Post manufacturing quality control consists of nutrient testing post processing to determine nutrient losses, long- and short-

term storage of foods to assess microbial stability and shelf-life losses of nutrients, as well as ongoing tracking of product stability in multiple environmental conditions (Zicker, 2008).

The research studies presented in this dissertation used semi-moist pet food and pet food kibbles as model food substrates to assess some novel food safety hurdles and mitigation strategies.

Food safety issues in semi-moist and low moisture foods

As mentioned earlier, semi-moist or intermediate moisture food products are below the minimum water activity for most bacteria, which require 0.90 a_w, but are susceptible to yeast and mold growth which leads to spoilage. Lowering the water activity is an essential processing step and the main microbial hurdle in intermediate moisture food products. However, setting the a_w at a certain level cannot by itself ensure shelf stability. Other factors and properties of food systems should be considered, and often additional measures must be taken to achieve the desired stability. Table 1.1 shows some of the common microorganisms growing in the a_w range of intermediate moisture foods (Beuchat, 1981).

Table 1.1. Microorganisms growing in the a_w range of intermediate moisture foods.

a_w	**Microorganisms generally inhibited by lowest a_w in this range**	**Examples of foods with a_w in this range**
0.80-0.84	Most molds (mycotoxigenic Penicillia), most *Saccharomyces* spp. (e.g., *S. baili*, *Debaryomyces*)	Most fruit juice concentrates, sweetened condensed milk, chocolate syrup, maple and fruit syrups, flour, rice, pulses containing 15-17% moisture, fruitcake, country-style ham, fondants, high-sugar cakes.
0.75-0.80	Most halophilic bacteria, mycotoxigenic Aspergilli	Jam, marmalade, marzipan, glazed fruits, some marshmallows.
0.65-0.75	Xerophilic molds (*Aspergillus chevalieri*, *A. candidus*, *Wallemia sebi*), *Saccharomyces bisporus*	Rolled oats containing about 10% moisture, grained nougats, fudge, marshmallows, jelly, molasses, raw cane sugar, some dried fruits, nuts.
0.60-0.65	Osmophilic yeasts (*Saccharomyces rouxi*), few molds (*Aspergillus echimulatus*, *Monascus bisporus*)	Dried fruits containing 15-20% moisture, some toffees and caramels, honey.

Microbial stability is also the primary criterion for the viability of an intermediate moisture food product under development. Inhibiting microbial growth on a given substrate is not achieved exclusively by lowering the a_w, but rather, it is a function of all contributing hurdles i.e., a_w, pH, temperature, oxidation reduction potential, preservatives, and existing microflora (Leistner and Gould, 2012). Numerous microorganisms of significance that cause spoilage have been shown to be able to grow at a_w in the range of 0.60 to 0.84 when other conditions are favorable (Table 1.1). Thus, additional precautions, besides the adjustment of a_w, must be taken to inhibit or limit the proliferation of these microorganisms in intermediate moisture food products.

The pathogenic microorganisms of major concern in foods are effectively inhibited by the reduction of a_w to the intermediate moisture zone. For example, the growth of Clostridia were prevented by reduced a_w regardless of storage temperature and pH (Roberts and Smart, 1976). However, because growth could potentially occur during formulation and storage prior to the reduction of a_w, good hygienic and manufacturing practices are essential. *Bacillus* spp. require a minimum a_w of 0.89 to 0.90 for growth (Jakobsen, 1985). At water activities found in intermediate moisture foods, *Salmonella* spp. cannot multiply due to a limit for growth of 0.95 a_w, but their resistance to heat is greatly increased, and they may persist in intermediate moisture foods for long periods (Corry, 1976). *Listeria monocytogenes* Pirie (Listeriaceae: Bacillales), can grow at considerably lower a_w with the reported limit of growth at 0.92 a_w (Cole et al., 1990, De Daza, 1991). The food pathogen able to grow at even lower a_w is *Staphylococcus aureus* Rosenbach (Staphylococcaceae: Bacillales), which has also been shown to grow at a_w above 0.84 to 0.85 if the pH is favorable (Pawscy and Davis, 1976). Formulation of intermediate moisture food products at the highest possible moisture content, for improved texture and palatability, requires additional measures for the inhibition of *S. aureus*. The same is true for molds. The most often encountered

molds are *Aspergillus* and *Penicillium* spp., which can grow at a_w above 0.77 to 0.85. The minimum a_w for mycotoxin production by these molds is usually higher. Several xerophilic and xero-tolerant molds can grow at a_w down to 0.62 to 0.64. Yeast growth is another potential problem with intermediate moisture foods. Compared with the most tolerant halophilic bacteria that can grow down to the a_w levels of saturated sodium chloride (i.e., a_w 0.75), osmophilic yeasts can grow at water activities down to 0.62 a_w (e.g., *Zygosaccharomyces rouxii* (Boutroux) Yarrow (Saccharomycetaceae: Saccharomycetales).

Good manufacturing practices, pasteurization of mixtures, and use of chemical preservatives, such as sulfites, benzoates, para-hydroxybenzoates, sorbates, and diethyl pyrocarbonate, are the usual control measures in intermediate moisture foods (Barbosa-Cánovas, 2020).

Apart from the above-mentioned food safety and spoilage issues of bacteria, molds and yeasts, another important issue for intermediate moisture foods during storage is infestation by mites. Mites are small arachnids (arthropods) that feed on stored food products causing economic losses and may also cause allergies to humans and pets (Vogel et al., 2015). They also act as vectors of other pathogens (Wisniewski, 1996). Storage mites (e.g., *Acarus siro* Linnaeus (Acaridae: Sarcoptiformes) and *T. putrescentiae*) feed on mold that grows on food (Sinha and Mills, 1968). Storage mites thrive in environments where there is moisture or increased humidity but are most frequently found in dry intermediate moisture food items such as flour, grains, dried fruits, cereal, and semi-moist dog and cat foods.

Mold contamination

Molds and mycotoxins affecting semi-moist foods

Fungi are widely distributed in the environment and are frequent contaminants of food and animal feed. They appear as spoilage-causing organisms under reduced values of water activity in intermediate (0.75-0.90 a_w) and low moisture (<0.75 a_w) foods, and foods with low pH values <4.0 (acidic foods). The minimum water activity limits for the growth of some of the common mold species found in intermediate moisture foods are summarized in Table 1.2 (Anthony and Fontana, 2007).

Table 1.2. Minimum water activity limits for the growth of common mold species found in intermediate moisture foods.

a_w	Common mold species surviving in intermediate moisture food
0.85	*Aspergillus clavatus*
0.84	*Byssochlamys nivea*
0.83	*Penicillium expansum, Penicillum islandicum, Penicillum viridicatum*
0.82	*Aspergillus fumigatus, Aspergillus parasiticus*
0.81	*Penicillum cyclopium, Penicillium patulum*
0.80	*Penicillium citrinum*
0.79	*Penicillum martensii*
0.78	*Aspergillus flavus*
0.77	*Aspergillus niger, Aspergillus ochraceous*
0.75	*Aspergillus restrictus, Aspergillus candidus*
0.71	*Eurotium chevalieri*
0.70	*Eurotium amstelodami*
0.61	*Monascus bisporus*

The most common species of fungi isolated from human foods belong to the genera *Aspergillus, Penicillium, Fusarium, Alternaria, Cladosporium, Mucor, Rhizopus*, *Eurotium* and *Emericella* (Samson et al., 2004; Pitt and Hocking, 2009). Species of genera *Aspergillus*, *Penicillium* and *Eurotium* are storage fungi that can develop at ≤0.85 a_w, so they can be isolated from spices, dried fruits and vegetables, pumpkin seeds, sunflower seeds, stored cereals, and similar products that are under the categories of intermediate or low moisture foods (Kocić-

Tanackov and Dimić, 2013). Species of the genera *Fusarium* and *Alternaria* are 'field' fungi and their development requires higher moisture content in the substrate and lower temperatures (Kocić-Tanackov and Dimić, 2013). These species are usually found in cereal grains and cereal products. Also, they are common causes of illness of fruits and vegetables in the field, in addition to species of the genera *Sclerotina, Bortrytis, Monilia, Rhizopus, Mucor,* and *Penicillium.* Fungi are common contaminants of meat and milk products during storage, with species of genera *Penicillium, Aspergillus, Cladosporium, Geotrichum, Mucor, Sporotrichum, Trichoderma* commonly isolated from these food groups (Kocić-Tanackov and Dimić, 2013).

Growth of fungi in food leads to food spoilage, causing economic losses due to food waste. On the other hand, toxin-producing species of genera *Aspergillus* (*A. carbonarius, A. flavus, A. ochraceus, A. oryzae, A. parasiticus, A. versicolor*), *Penicillium* (*P. nordicum, P. expansum, P. viridicatum, P. verrucosum*), *Fusarium* (*F. culmorum, F. graminearum, F. oxysporum, F. verticillioides, F. proliferatum*), *Alternaria* (*A. alternata, A. solani, A. brassicae, A. tenuissima, A. tomato*) as well as teleomorphs of the class *Ascomycetes* (*Petromyces alliaceus, Emericella nidulans,* etc.*)* can biosynthesize toxic secondary metabolites namely mycotoxins. Examples of mycotoxins are aflatoxins, ochratoxin A, sterigmatocystin, patulin, fumonisins, zearalenone, deoxynivalenol, alternariol, alternariol monomethyl ether, and tenuazonic acid (Samson et al., 2004; Pitt and Hocking, 2009). Intake of mycotoxins by animals and humans causes intoxication called mycotoxicosis. Mycotoxicosis leads to acute and chronic toxicity (cytotoxicity, hepatotoxicity, neurotoxicity, teratogenicity, mutagenicity, and carcinogenicity) (Janik et al., 2020). At a cellular level, mycotoxins react with nucleic acids and inhibit the biosynthesis of macromolecules DNA and RNA, or act on structures and functions of biological membranes or impair the energy metabolism (Wang and Groopman, 1999; Diaz, 2005).

Throughout history there have been mass poisoning of humans and animals associated with consumption of food contaminated with fungi and mycotoxins (Mazumder and Sasmal, 2001). One of the first known mycotoxicosis reports was ergotism caused by ergot alkaloids. Ergotism was responsible for the deaths of thousands of people in medieval Europe (Mazumder and Samsal, 2001). In the 20th and 21st century, the appearance of mycotoxicosis described as a disease of horses and pigs in the USA which was associated with the intake of rye was contaminated with *Fusarium graminearum*. Also, the disease stachybotryotoxicosis of horses was reported in the USSR, and in Slovakia and Hungary for sheep. Disease reports for facial eczema of sheep in New Zealand, and liver tumors induced by 'yellow rice toxins' in Japan were reported after the World War II. The alimentary toxic aleukia in Siberia (1913), the Balkan endemic nephropathy (1952), and human aflatoxicosis in Kenya (2004) were reported. However, mycotoxins and mycotoxicosis did not receive much attention until 1960 when the 'X' disease of turkeys, ducklings and pheasants caused a great economic loss in England and led to the discovery of the causative mycotoxin, described as aflatoxin. It was named after the species which synthesized it, *A. flavus*, isolated from peanut flour fed to poultry (Wang and Groopman, 1999; Samson et al., 2004; Diaz, 2005;). Today there are more than 400 types of mycotoxins, and with the development of analytical methods the number is steadily increasing. However, only a few mycotoxins are well described in toxicology (aflatoxins, ochratoxin A, *Fusarium* toxins), and the aflatoxins are the most studied. Even though rapid technological advances during the last few decades have allowed the introduction of new technologies in food production in order to produce healthy, nutritious and safe products, the occurrence of fungi and mycotoxins in food products is still a concern. Numerous studies have confirmed their presence in almost every type of food product (Logrieco and Visconti, 2004; Weidenborner, 2008) and in animal feed (Whitlow et al., 2002).

Molds and mycotoxins also affect pet food. A study by Beuno et al. (2001) identified commonly occurring molds in 21 pet foods. These included dry dog kibbles across 8 commercial brands produced in Argentina. Molds were comprised mainly of *Aspergillus,* followed by *Rhyzopus* and *Mucor* spp., and *A. flavus* was found in 14 of 60 pet food samples purchased in Portugal (Martins et al., 2003). In another study Scudamore et al. (1997) identified significant growth of *Aspergillus* spp. in commercial pet food with 20-25% moisture content after a 4-week incubation. Although the presence of toxigenic fungi does not necessarily result in mycotoxin production, preventing fungal growth in pet food can certainly minimize the risk of mycotoxicosis. Fungal contamination can lead to economic losses associated with nutrient and palatability reduction, and the presence of mycotoxins affects both animal and human health (Savi et al., 2015). *Aspergillus flavus* is the most reported in pet food and responsible for the production of aflatoxins.

Aflatoxins are a group of mycotoxins produced by *Aspergillus* spp., mainly *A. parasiticus* and *A. flavus* (Moss, 1996). They are common fungal contaminants of corn, peanuts, cottonseed, tree nuts, wheat, and rice. Aflatoxins B1, B2, G1, and G2 are the four naturally occurring forms of aflatoxins, with aflatoxin B1 the most potent, prevalent, and carcinogenic (IARC-WHO, 1993). Aflatoxins are hepatotoxic and carcinogenic. Dogs are extremely sensitive to this group of toxins, with the liver being their main target (Silva et al., 2018). Dogs exposed to 0.5-1 mg of aflatoxin/kg of body weight typically die within days, exhibiting vomiting, depression, polydipsia, polyuria, and hepatitis (Leung et al., 2006).

In the US aflatoxicosis related illnesses in dogs and *Aspergillus flavus* contaminated dog food recalls were reported in 2005. More recently, in 2020, 28 deaths and 8 illnesses were reported in dogs that consumed the recalled Sportmix™ pet food product that was contaminated with aflatoxin. Pet food recalls by Sunshine Mills also happened in 2020, due to aflatoxin contamination

from corn that was used as ingredient in the pet food (FDA, 2021). Currently the FDA has an established action level of 20 ppb for aflatoxin in pet foods. Many of the recalled products contained >400 ppb aflatoxin (FDA, 2021). Thus, there is a need to control or reduce toxigenic *A. flavus* contamination.

Mold inhibitors commonly used in the food industry

The most commonly used chemical preservatives to prevent mold spoilage in intermediate moisture foods include: (i) propionates (calcium or sodium propionate), (ii) sorbates (sorbic acid and potassium sorbate), (iii) benzoates, (iv) parabens (methyl and propyl), and (v) acetic acid (Pyler and Gorton, 2008). Calcium and sodium propionate are the neutral salts of propionic acid. Propionate is a naturally occurring byproduct of the *Propionibacterium* spp. found in Swiss cheese. Due to their lack of activity against yeast, propionates are the most widely used antimicrobial and mold inhibitor in yeast-raised baked foods (Pyler and Gorton, 2008). Propionic acid is industrially produced by the hydrocarboxylation of ethylene and the aerobic oxidation of propionaldehyde (Samel et al., 2005). Calcium propionate and sodium propionate are produced by the acid-base reaction of calcium carbonate and sodium hydroxide with propionic acid. Sorbic acid, and its sodium and potassium salts, are effective against yeasts and molds. Consequently, since these products can inhibit yeast fermentation, sorbates are applied to intermediate moisture food products as encapsulates sprayed onto the product as an aerosol or incorporated into packaging material. Sorbic acid was first isolated from the berries of Mountain Ash (*Sorbus spp.*) trees (Bullerman, 2000) and is commercially prepared by synthetic procedures, such as the condensation of crotonaldehyde and ketene (Weissermel and Arpe, 2003). Benzoates (sodium benzoate) are inhibitory to yeast and mold and most commonly used to delay spoilage of high acid fillings, fruits and jams (Pyler and Gorton, 2008). Sodium benzoate is manufactured by the

neutralization of benzoic acid with sodium hydroxide (Wibbertman et al., 2000). Benzoic acid naturally occurs in cranberries, prunes and cinnamon. Parabens are related to benzoic acid-esters of para-hydroxybenzoic acid. Because they share benzoic acid's ability to inhibit yeast and mold activity, parabens are typically used in cereal and potato-based snacks (Pyler and Gorton, 2008). Although these are effective mold inhibitors, they are considered to be 'synthetic' chemical additives. In recent times, consumers have preferred natural, clean label ingredients in their foods. Thus, there has been growing research to investigate clean-label mold inhibiting components to improve shelf life of intermediate moisture foods. Table 1.3 summarizes some of the previous research that investigated natural/clean label ingredients to inhibit mold growth in semi-moist foods (Albers-Nelson, 2010; Kocić-Tanackov and Dimić, 2013).

Table 1.3. Natural/clean label ingredients inhibiting mold growth in foods.

Mold/Mycotoxin	**Clean-label mold inhibitor**	**Effective concentration**	**Food substrate**	**References**
Environmental mold	Vinegar (acetic acid)	0.1-0.3%	Wheat bread	Dubois, 1983; Bullerman, 2000
	Prune juice concentrate (malic, benzoic, salicylic acids)	9-12%	Wheat bread	Sanders, 1990; 1991
	Raisin paste concentrate, raisin juice concentrate (propionic acid, tartaric acid)	5-10%	Wheat bread	Fagreel, 1992; Label, 2000; Bullerman, 2000
	Cinnamon oil (cinnamaldehyde)	1-2% dry spice	Wheat bread	Bullerman, 2000
	Clove oil (eugenol)	1% dry spice	Wheat bread	Bullerman, 2000
	Natamycin	7-20 ppm	Wheat bread	Bullerman, 2000
Alternaria alternata	Cassia	100 mg/kg	Cherry tomato	Feng and Zheng, 2007
Aspergillus flavus	Anise, green mint	2%	Wheat grains	Soliman and Badeaa, 2002

	Allyl isothiocyanate	2 µL/gas phase	Rye bread, 'hot dog' bread	Nielsen and Rios, 2000
	Cinnamon	2%	Wheat grains, orange fruits	Soliman and Badeaa, 2002
	Cinnamon, clove, bay laurel, thyme, rosemary, sage	270 µL/L gas phase	Black rye bread	Suhr and Nielsen, 2003
	Thyme	2%	Wheat grains	Soliman and Badeaa, 2002
	Lemon grass, orange, mustard	135 µL/L gas phase	Black rye bread	Soliman and Badeaa, 2002
	Thyme, clove, savory	300 mg/kg, 500 mg/kg	Tomato ketchup	
Aspergillus ochraceus, Aspergillus parasiticus	Anise, cinnamon, thyme, green mint	2%	Wheat grains	Soliman and Badeaa, 2002
Botrytis cinerea, Colletotrichum coccodes	Cinnamon	500 mg/kg	Cherry tomato, pepper	Tzortzakis, 2009
Fusarium verticillioides	Anise, cinnamon, thyme, green mint	2%	Wheat grains	Soliman and Badeaa, 2002
Eurotium repens	Cinnamon, clove, bay laurel, rosemary, sage	270 µL/L gas phase, 50 µL/gas phase	Black rye bread, biscuits analogue	Suhr and Nielsen, 2003
	Thyme, lemon grass, orange, mustard	135 µL/L gas phase, 50 µL/gas phase	Black rye bread, biscuits analogue	Suhr and Nielsen, 2003
Penicillium commune	Allyl isothiocyanate	2 µL/L gas phase	Rye bread, hot dog bread	Nielsen and Rios, 2000
Penicillium corylophilum	Cinnamon, cloves, bay laurel, thyme, orange, rosemary, sage	270 µL/L gas phase	Black rye bread	Suhr and Nielsen, 2003
	Lemongrass, mustard	135 µL/L gas phase	Black rye bread	Suhr and Nielsen, 2003
	Allyl isothiocyanate	2 µL/L gas phase	Rye bread, hot dog bread	Nielsen and Rios, 2000
Penicillium expansum	Cinnamon	2.0% (v/v)	Orange peels	Xing et al., 2010
Penicillium roqueforti	Cinnamon, clove, lemongrass, bay laurel, thyme,	270 µL/L gas phase	Black rye bread	Suhr and Nielsen, 2003

	orange, rosemary, sage			
	Mustard	135 μL/L gas phase	Black rye bread	Suhr and Nielsen, 2003
	Allyl isothiocyanate	2 μL/L gas phase	Rye bread, hot dog bread	Suhr and Nielsen, 2003
Rhizopus nigricans	Cinnamon	2.0% (v/v)	Orange fruits	Xing et al., 2010
Sclerotina sclerotiorum	Dill, oregano	3.2 μg/mL	Tomato fruits	Soylu et al., 2007
Monilia fructigena, Phlyctema vagabonds, Botrytis cinerea, Penicillium expansum	Eugenol	2 mg/mL	Apple fruits	Amiri et al., 2008
Aflatoxin B1	Cinnamon, clove	1000 mg/kg	Corn grains	Sinha et al., 1993
Aflatoxin B1, G1	Basil	100 mg/g	Melons, peanuts, sorghum, corn	Atanda et al., 2007

Mode of action of natural mold inhibitors

The chemical structure of the natural plant essential oil components and their antifungal properties are related. The presence and position of the hydroxyl group in the molecule, the presence of the aromatic ring, solubility in fats and spatial orientation affect the antifungal activity (Raccach, 1984; Veluri et al., 2004). Compounds containing aromatic rings include phenolic compounds and are characterized by high antimicrobial activity (Farag et al., 1989; Decker, 1995). Also, presence of an alkyl group on the benzene ring of phenols or guaiacol (Kurita et al., 1981), acetate groups (for example, geranyl-acetate has a stronger antimicrobial activity than geraniol, and bornyl-acetate than borneol) (Dorman and Deans, 2000), oxygen in monoterpenes and their carbonylated compounds (Naigre et al., 1996) enhance the antifungal activity of the components. Phenolic components (carvacrol, thymol, eugenol etc.) exhibit the strongest antifungal and antimycotoxigenic activity, followed by alcohols, aldehydes, ketones, esters and hydrocarbons

(Kurita and Koike, 1983). A possible mechanism of action for plant essential components on the growth of fungi has been reported in several studies. It is generally accepted that the essential oil components act on the functionality and the structure of the cell membrane (Viuda-Martos et al., 2008). Low concentrations result in changes of the cell structure, inhibiting respiration and changing the permeability of the cell membrane, whereas high concentrations lead to severe membrane damage, loss of homeostasis and cell death (Carson et al., 2002). Conner and Beuchat (1984) suggested that the antifungal activity is the product of essential oil components' interaction with enzymes responsible for energy production and the synthesis of structural compounds of the cell. On the other hand, Omidbeygi et al. (2007) suggested that the essential oil components pass through the cell membrane, integrate with membrane enzymes and proteins of membranes, causing a loss of macromolecules from the interior of the cell, and thereby leading to changes in the cell and ultimately to its death.

Mite infestation

Mite infestation in stored food products

Mites are common pests of stored cereals and oilseed. Storage mites belonging to the Glycyphagidae and Acaridae families including *Tyrophagus*, *Acarus*, *Lepidoglyphus* and *Glyciphagus* are considered to be important pests in stored foods and a source of environmental allergens for humans and pets like dogs with atopic dermatitis (Vollset et al., 1986; Nuttall et al., 2006). These mites are often found in stored hay, straw, grain, and dry feed stuffs. In the UK, surveys have detected their presence in over 80% of stores, with *Acarus siro*, *Lepidoglyphus destructor*, *Tyrophagus longior*, and *Tyrophagus putrescentiae* in order of predominance (Lynch et al., 1991; Prickett, 1997). Surveys from other parts of the world have found *L. destructor*, *T.*

putrescentiae and *Tyrophagus* spp. to be the predominant species, with *A. siro* occurring less frequently than in temperate regions (Mahmood, 1992; Emmanouel et al., 1994; Haines, 1997).

Among astigmatid mites, *Tyrophagus putrescentiae* is a cosmopolitan species commonly encountered infesting a large number of intermediate and low moisture foods including food grains and stored food with a high fat and protein content, such as dried eggs, dried bananas, cheeses, ham, fishmeal, wheat spillage, oats, flour, and different kinds of nuts (Hughes, 1976; Sinha, 1979). It causes serious economic losses as well as reducing nutrient content and germination ability (Krantz, 1955). In addition, the mite is responsible for allergic diseases among farmers and food industry workers handling heavily infested stored products (Hughes, 1976) and causes acute enteritis (Hughes, 1976) and systemic anaphylaxis (Matsumoto et al., 1996) when contaminated food is ingested. The mite also acts as a carrier of bacteria and toxigenic fungi such as *Aspergillus* spp. in stored grain kept under warm and moist conditions (Franzolin et al., 1999). When food products, including pet food, are stored at homes by consumers, they are susceptible to mite infestation from house dusts which can harbor some storage mites. Brazis et al. (2008) observed that two out of ten different brands of sealed commercial dog foods contained storage mites, and upon storage at optimal temperatures and humidity, nine out of ten products contained storage mites.

T. putrescentiae is a small mite species (0.28-0.41 mm) with a translucent body and virtually colorless chelicerae and limbs. The mite's life cycle requires 2–3 weeks under ideal environmental conditions (23°C and a relative humidity of 87%). According to Bahrami et al. (2007), typical development times for eggs, larvae, and the two nymphal stages are 2, 3 and 5 days, respectively at a temperature of 25°C and 70% RH. There are different reports on longevity of adult mites depending on temperature, relative humidity, and food source (Boczek, 1991).

Lifespan of the mites reared on wheat germ at 25°C and 85% RH was about 60 days. In terms of food impact on longevity, it was up to 120 days for mites reared on wheat germ, 80 days on pumpkin seeds, 75 days on powdered milk and 25 days on rolled oats. A female mite reared on wheat germ or yeast can produce 500 eggs (Boczek, 1991).

Methods to control mite infestation

Traditionally, control of these mites depends on chemical methods such as fumigation with methyl bromide, spraying with organophosphorus compounds, or treatment with pesticides like benzyl benzoate and repellents like DEET (N, N-diethyl-m-toluamide) because ecological control methods with high humidity and temperature cause alterations in food quality. Residual pesticides are mainly used to treat the structure of buildings, and some are applied directly to the food commodity. Methyl bromide, though an effective fumigant used to control storage mites like *T. putrescentiae*, has been phased out in industrialized nations across the world including the US due to its ozone-depleting nature. Numerous efficacy tests have been performed with experimental and formulated acaricides against mold mites in the context of agricultural and industrial settings (Kim et al. 2003; Collins et al., 2006; Palyvos et al. 2006; Hubert et al. 2007; Jeong et al. 2008; Stara et al. 2011). They leave behind some residue on the treated commodity, and repeated use of these chemicals have resulted in the development of resistance by the mites (Szlendak et al., 2000). These chemicals have had undesirable effects on non-target organisms and have led to environmental and human health concerns (Hays et al., 1991). These problems have highlighted the need for the development of new strategies for targeted storage mite control, preferably using safe, non-toxic, and natural compounds. Some of the previous research studies that have investigated natural/clean label components to inhibit *T. putrescentiae* infestation is summarized in Table 1.4 (Collins, 2006).

Table 1.4. Natural/GRAS (generally recognized as safe) ingredients evaluated in food substances to mitigate *T. putrescentiae* infestation.

Natural/GRAS component	**Doses**	**Substrate**	**Result**	**References**
Commercial product of neem (Fortune AZA)	25-100, 20-80 ppm	Wheat, Oilseed	20-95%, 0-65% population inhibition	Collins et al., 2001; Collins, 2003
Petroselinum sativum (parsley)	8.4%, 16.8%	Food	100% mortality	Czajkowska, 1971
Ethanol extracts of neem, Ethanol extracts of tarragon	17-80%, 18%	Food	Decreased longevity and fecundity	Czajkowska, 2002
Neem oil, Neemark, Neem cake	0.4%	Wheat	93%, 85%, 81% decrease in eggs	Gulati, 1998
Caraway extract, Fenugreek extract, Caraway + fenugreek extracts, Lupin extract	12, 12, 100, 100 ppm	Flour	100% mortality	Afifi and Hafez, 1988
Commercial product of benzyl benzoate (Acarosan spray)	50-200 ppm	Wheat	2-99% population inhibition	Collins et al., 2001
Linalool	0.1-100 ppm	Wheat	0% population inhibition	Collins et al., 2001
Eucalyptus powder, Mentha powder, Curcuma rhizomes	5-75%, 5-75%, 0.1-10%	Flour	47-98%, 74-97%, 92-100% decrease in egg production	Gulati and Mathur, 1995
Curry powder + lemon/pepper seasoning + mace + sage, Clove, Almond, Sassafras, Black pepper	2%, 0.1%, 0.01%, 0.01% and 0.001%, 0.001%	Food	Feeding deterrent effects, 92%, 82%, 96% and 94%, 74% growth inhibition	Rodriguez et al., 1979
Azadirachtin	10-10,000 ppm	Diet	Low immature mortality	Sanchez-Ramos and Castanera, 2003
1,8-cineole, Limonene, Essential oil of Pinus pinea	6, 8, 8 µL	Fumigant	100% mortality	Macchioni et al., 2002
Lemongrass + thyme white oil	25.5 mg/cm^2	Fumigation	100% mortality	Kim et al., 2003a

Cinnamaldehyde, Cinnamyl alcohol, Salicylaldehyde	50.9 mg/cm^2	Fumigant	100% mortality	Kim et al., 2004
Acetyleugenol, Eugenol, Isouegenol, Methyleugenol	25.5 mg/cm^2	Fumigant	100% mortality	Kim et al., 2003b
Eucalyptol, Fenchone, Linalool, α-terpinene, γ-terpinene, Menthone, Pulegone	21.5, 13.5, 11.2, 43.4, 42.6, 6.2, 4.7 µL/L	Fumigant	90% mortality	Sanchez-Ramos and Castanera, 2001
Propylene glycol (1,2-propanediol), Lard, Ethoxyquin, Butylated hydroxytoluene (BHT)	10-100%, 10-100%, 1-10%, 10%	Dry cured ham pieces	Decrease in population growth, inhibition of reproduction	Abbar et al., 2016
Carrageenan+propylene glycol alginate+40% propylene	Coating	Whole aging ham	Decrease in population growth	Abbar et al., 2016

Utilizing mitigation strategies along with good sanitation practices in the industry and storage spaces help in developing effective integrated pest management for mites in these areas.

Contamination of food contact surfaces

Contaminants on surfaces in the food industry

Contaminated surfaces can transmit and perpetuate pathogens to food in industrial and domestic food handling environments. Exposure to pathogens on surfaces may take place either directly by contact with contaminated objects or indirectly via aerosols originating from the surface. Various bacteria of public health significance, including *Salmonella, E. coli*, and *Listeria*, can survive on hands, sponges, clothes, utensils, and currency for hours or days (Scott and Bloomfield, 1990). Pathogenic bacteria may remain on equipment surfaces even after disinfection procedures are applied, increasing the risks associated with the transmission of diseases (Dunsmore et. al., 1981). Not only bacteria, but mold spores from the environment may also remain on equipment surfaces, on the floor of food manufacturing facilities, on automobile tires etc. and

eventually find their way to contaminate foods. Therefore, domestic and industrial food handling environments can be important sources of foodborne pathogens and spoilage organisms including bacteria and molds. Further complicating biological hazard control on these surfaces is the potential formation of biofilms. Biofilms are formed when individual bacterial cells adhere and embed in an extracellular polymeric substance on surfaces such as processing equipment providing a defense mechanism (McLandsborough et al., 2006). Pathogenic bacteria, including *Salmonella* spp. and *Listeria monocytogenes*, may form biofilms in processing environments (McLandsborough et al., 2006). Their extracellular polymeric matrix is difficult to penetrate for sanitizing, for example, *Salmonella* spp. has been shown to maintain presence on dry surfaces for up to 4 weeks through a biofilm (Habimana et al., 2010).

Sanitizers used to decontaminate surfaces

Preventive measures utilized by food industry have included coating of surfaces to limit the establishment of vegetative cells or biofilms (Jullien et al., 2003). Huss et al. (2015) and Schumacher et al. (2016) demonstrated that liquid decontamination of animal food manufacturing equipment appears effective but were not very practical or easy to implement. Generally, a water activity level >0.87 a_w is required for growth of most bacterial pathogens of concern including *Salmonella*, so introducing a water-based sanitizer would raise the a_w to levels that allow for *Salmonella* growth. Chlorine and chlorine derivatives have been used as a broad-spectrum bactericidal, fungicidal, sporicidal, tuberculocidal, and virucidal control within food manufacturing facilities. Hypochlorite and sodium chlorite can be effective detergents and sanitizers and are known to penetrate biofilms developed by *Salmonella* (Joseph et al., 2001; Ramesh et al., 2002). However, hypochlorite solutions may produce the carcinogens bischloromethyl ether when in contact with formaldehyde (Gamble, 1977), and trihalomethane

when in contact with hot water. Because of their potential impact on human health, chlorine and its derivatives must be rinsed from surfaces prior to manufacturing food for consumption by humans or pet animals.

Quaternary ammonium compounds are known to be effective sanitizers of fungi, bacteria, and non-enveloped viruses. Their true advantage is an ability to effectively sanitize *Salmonella* spp. biofilms and because the presence of organic matter is not as inhibitory to their action as it is to other sanitizers (Betty et al., 2005). These compounds have been demonstrated to reduce *Salmonella* spp. by 2 to 3 CFU/cm^2 log on galvanized steel (Ramesh et al., 2002). Research has also demonstrated their effectiveness to sanitize stainless steel contaminated with *Listeria* (Pan et al., 2006; Ronner and Wong, 1993) and rubber contaminated with *Salmonella* (Ronner and Wong, 1993).

Due to their effectiveness and positive consumer perception of being chemical-free/non-toxic, acid-anionic sanitizers including acetic, benzoic, and propionic acids, have been favored as antimicrobials in food processing facilities. Their mode of action includes acidifying the cytoplasm and disrupting cell membrane organization, which is highly effective for *Salmonella* destruction in food manufacturing facilities (Ricke, 2003; Carrique-Mas et al., 2007). Due to their effectiveness and relative safety, organic acid salts have also been used as surface treatment of foods to prevent microbial growth and as a preservative in finished foods (Smulders, 1995; Samelis et al., 2005). For example, sodium bisulfate, a dry GRAS acidulant, has been demonstrated to prevent *Salmonella* cross-contamination when used as a coating on pet food kibbles (Jeffrey, 2016). Recent research has utilized antimicrobial properties of medium chain fatty acids for reduction of *Salmonella* Typhimurium in animal feed (Cochrane et al., 2016) and pet food (Dhakal and Aldrich, 2020). Other fatty acids have been used for inclusion in poultry diets for salmonellosis

control. Believed antimicrobial modes of action include disruption of the cell membrane and other essential functions (Desbois and Smith, 2010). Cochrane (2016) demonstrated efficacy of medium chain fatty acids on reduction of porcine epidemic diarrhea virus. There has been growing interest in investigating novel food-safe sanitizers to decontaminate food contact surfaces in manufacturing facilities.

Rationale for this research

According to an annual poll conducted by the Center for Food Integrity, consumers have less confidence in the safety and quality of the food supply and are demanding more all-natural and minimally processed foods with less synthetic chemical additives (Andrews and Withey, 2012). Consumers also have increased interest in organic foods because they are often viewed to be healthier, better tasting, or fresher than conventional products (Wier and Calverley, 2002). However, though free of synthetic chemicals, organic and all-natural foods are not exempt from bacterial contamination and may require the addition of antimicrobials to ensure their safety. All-natural antimicrobials including those derived from plants, animals and bacteria have been shown to be effective at increasing the safety of food products by destroying or limiting the growth of bacterial pathogens (Juneja et al., 2012; Davidson et al., 2013). Liquid smoke is approved by the US-FDA as a "Generally Recognized as Safe" (GRAS) substance and can be included in human and pet foods with no specific limit and the flavor of liquid smoke is widely likeable in these foods as well. With the U.S. Patent 4377187, the use of liquid smoke to impregnate fibrous cellulosic food/meat casings to prevent mold growth was found to be effective by Chiu (1983). Wendorff et al. (1993) evaluated the effects of liquid smoke on the growth of *Aspergillus oryzae, Penicillium camemberti,* and *Penicillium roqueforti* on cheddar cheese. They found that liquid smoke applied to the surface of cheese inhibited growth of *A. oryzae and* increased the lag period of *P.*

camemberti and *P. roqueforti*. Liquid smoke and its fractions have been found to be effective against pathogenic bacteria like *Listeria monocytogenes* and *Staphylococcus aureus* (Sunen et al., 2003; Lingbeck et al., 2014) in meat and fish products, but their effects against fungi or molds in food substrates have not been studied. Milly et al. (2005) evaluated the phenol and carbonyl fractions of liquid smoke in nutrient broth against the mold *A. niger* and reported their minimum inhibitory concentrations to be in the range of 1.5% to 5%. Beyond these we could not find any available scientific literature on the antimycotic potential of liquid smoke in any food matrix against common storage molds such as *Aspergillus* and *Fusarium* spp. which produce mycotoxins (aflatoxin and deoxynivalenol, respectively) in food and feed.

There is no available information on the acaricidal potential of liquid smoke against storage mites. Eischen et al. (2004) tested wood smokes from 40 different plants on *Varroa* mites (bee mites) and found two of them to be effective. To our knowledge, there are no studies that tested liquid smoke on stored food mite species including *T. putrescentiae.* Also except for a very limited number of studies like the one by Ernst et al. (2007), who reported coating pet food with conjugated linoleic acid inhibited *T. putrescentiae* growth/infestation, and Manu et al. (2021), who reported a mixture of three short-chain fatty acids $C_{8,}$ $C_{9,}$ C_{10}, and the sesquiterpene ketone nootkatone coated on ham cubes repelled *T. putrescentiae*, we could not find any published literature that tested inhibition or repellency effect of food-safe components on *T. putrescentiae* infestation in semi-moist foods. Evaluating the antifungal and acaricidal potential of liquid smoke can help diversify its use in protecting intermediate moisture foods like semi-moist pet food from mold contamination and mite infestation.

HMTBa (2-hyroxy 4-methlythio butanoic acid) is an organic acid and a food-safe methionine hydroxy analogue. It has been used as a methionine precursor in animal feed due to its

unique chemical structure that allows protection from some of the microbial degradation in the rumen gut. HMTBa also provides acidifying effects of organic acids. A HMTBa-based organic acid mixture "Activate DA" has been used as a supplement applied to premixes and finished feeds. Another HMTBa-based organic acid mixture "Activate WD-MAX" has been used for acidification of drinking water for poultry, making the drinking water a less favorable environment for pathogen growth and it is shown to play an important role in the destruction of harmful microorganisms in the gut that could affect the birds' performance. There is limited knowledge on the application of these organic acid mixtures in low moisture foods like dry pet food kibbles and food contact surfaces as an antimicrobial to enhance food safety. Investigating their potential as an antimicrobial and antifungal agent in low moisture foods like pet food kibbles and as food-grade sanitizers on food contact surfaces can diversify their application in high value food products (human and pet foods) and safe, non-toxic addition to conventional sanitizing of food processing spaces.

Liquid smoke

Application of liquid smoke in the food industry

Traditional smoking of foods, especially meats, has been used as a preservation technique for centuries. Wood smoke, in addition to preserving food quality with its antioxidant and antimicrobial properties, also imparts a desirable color, flavor and aroma to smoked foods. Application of liquid smoke requires less time than traditional smoking, is more environmentally friendly, and eliminates potentially toxic compounds while still imparting the desired flavors and aromas of traditional smoking. Use of condensates of liquid smoke allows the processor to control the concentration of smoke being applied more readily than generating smoke by burning of wood (Sunen et al., 2001). Liquid smoke is traditionally applied to meat, fish and poultry and it has also

been used to impart flavor to non-meat items such as cheese, tofu and even pet food. Because the smoke flavor is concentrated, application of liquid smoke is best suited for use in marinades, sauces or brines or topically to processed meat items such as hot dogs, sausage, ham and bacon (Rozum, 2009).

The invention of liquid smoke is credited to E.H. Wright, a late 19th century Kansas pharmacist. The product was initially used in a domestic setting for the curing of bacon and hams, as well as for the flavoring of products such as stews and baked beans. Smoke flavors have been produced on a large scale since the 1970's and are now progressively replacing traditional smoking methods. Liquid smoke methods have gained popularity over traditional methods due to several advantages like ease and speed of application, uniformity of product, final product reproducibility, functionality and reactionary properties, and cleanliness of application. Since its introduction into the food industry, liquid smoke has taken on several functionalities independent of smoking meats. With the US Patent 5637339, liquid smoke may be fractionated, de-phenoled (de-flavored), and further refined to yield a product applicable as a functional ingredient in a variety of food applications, including dairy, vegetable, coatings, and pastry industries (Moeller, 1997).

Generation of liquid smoke

Liquid smoke is produced by condensing wood smoke created by the controlled, minimal oxygen pyrolysis of sawdust or wood chips. The wood is placed in large retorts where intense heat is applied, causing the wood to smolder, releasing the gases seen in ordinary smoke. These gases are quickly chilled in condensers, which liquefies the smoke. The liquid smoke is then forced through refining vats, and then filtered to remove toxic and carcinogenic impurities. Finally, the liquid is aged for mellowness. Factors influencing the flavor and antimicrobial properties of liquid smoke include the temperature of smoke generation, moisture content of the wood as well as the

type of wood used to generate the smoke (Simko, 2005). Common woods include hickory and mesquite, but liquid smoke has also been prepared from rice hulls (Kim et al., 2011; 2012), coconut shells (Zuraida et al., 2011) and pecan shells (Van Loo et al., 2012). In general, woods used to generate liquid smoke are roughly comprised of 25% hemicellulose, 50% cellulose, and 25% lignins (Simko, 2005). Pyrolysis occurs in four stages starting with water evaporation, followed by decomposition of hemicelluloses, cellulose decomposition and finally decomposition of lignins. Pyrolysis of hemicellulose and cellulose occurs between 180°C and 350°C and produces carboxylic acids and carbonyl compounds while lignins are pyrolyzed between 300°C and 500°C and generate phenols (Ramakrishnan and Moeller, 2002; Simko, 2005). Compounds present in liquid smoke, including phenols, are responsible for the smoke flavor and smoky aroma while carbonyl compounds impart a sweet aroma and color to smoked meat products. In addition to carbonyls, acids, and phenols, pyrolysis of wood often generates unfavorable compounds such as polycyclic aromatic hydrocarbons. Although they are toxic, they have low water solubility which allows liquid smoke manufacturers to easily separate out these compounds from their finished products using phase separation and filtration techniques.

Composition of liquid smoke

Baltes et al. (1981) found the major proportion of commercial full-strength liquid smoke to be composed of water (11-92%), tar (1-17%), acids (2.8-9.5%), carbonyl containing compounds (2.6-4.6%) and phenol derivatives (0.2-2.9%). Phenolic compounds contribute to smoke flavor and color of liquid smokes, and also have antibacterial and antioxidant properties (Clifford et al. 1980; Maga 1987; Varlet et al. 2010). Carbonyl-containing compounds impart sweet or burnt-sweet aroma and tend to soften the heavy smoky aroma associated with phenolic compounds with some ‘typical smoke-cured’ aroma and flavors (Fujimaki et al. 1974; Kim et al. 1974; Kostyra and

Barylko-Pikielna, 2006). Furthermore, carbonyl-containing compounds are involved in textural changes in smoked food caused by interaction with proteins and contribute to the golden-brown color of smoked products due to reaction with amino acids, and the formation of Maillard reaction products (Varlet et al. 2007).

The composition of wood smoke is directly related to the type of wood source. Generally, trees are composed of approximately 45% cellulose, 20-30% lignin (polyphenol), and 25-35% hemicellulose. All wood sources yield smoke that is a very complex mixture of over 400 different compounds including alcohols, carbonyls, esters, furans, lactones, phenols, and others. Identification of compounds present in wood smoke is possible through the use of gas chromatography-mass spectrometry (GC-MS) methods (Guillen and Ibargoitia, 1999). Some of the over 400 volatiles identified in liquid smoke are summarized in Table 1.5 (Maga, 1988). This list includes only a few of the 48 acids, 22 alcohols, 131 carbonyls, 22 esters, 46 furans, 16 lactones, 75 phenols, and 50 miscellaneous compounds known to exist in liquid smoke (Maga, 1988).

Table 1.5. Compounds primarily identified in wood smoke.

Group	Compounds
Alcohols	Methyl, Ethyl, Propyl, Isopropyl, Isobutyl, Propan-2-on-ol, Cyclohexanol, Benzyl, Butan-2-on-1-ol, Amyl
Esters	Methyl Formate, Methyl Acetate, Methyl Propionate, Methyl Butyrate, Methyl Crotonate, Ethyl Benzoate, Methyl Valerate, Methyl Isobutyrate, Cresyl Acetate, Methyl Palmitate
Acids	Formic, Acetic, Glycolic, Propionic, Isobutyric, Benzoic, Sorbic, Isovaleric, 3-Butenoic, Valeric
Carbonyls	Methanal, Propanal, Acetone, Acetol, Diacetyl, Hydroxyacetaldehyde, Pentanone, Cyclopentanone, Benzaldehyde, Hexanal
Lactones	Butyrolactone, Butenolide, Angelica Lactone, Hydroxyvalerolactone, 2-Methyl-2-Butenolide, Methylvinyl-2-Butenolide, 2,3-Dimethyl-2-Butenolide, 2,3,4-Trimethyl-2-Butenolide, Crotonolactone, 4-Ethyl-2-Methyl-2-Butenolide
Furans	Furfuryl Alcohol, Furans, 2-Methylfuran, 3-Acetylfuran, Propylfuran, Amylfuran, Benzofuran, 2-Furoic Acid, 2-Furfural, 5-Methylfurfural

Phenols	Diethylphenol, 4-Butylphenol, 4-Propylphenol, 4-Vinylphenol, 3-Methoxyphenol, Guaiacol, Pyrocatechol, Isoeugenol, 2,6-Xylenol, Cresol
Miscellaneous	Pyrazine, Pyrrole, Pyridine, Maltol, Ethanediol, Toluene, Styrene, Benzene, Indene, Naphthalene

Antimicrobial activity of liquid smoke

The phenols and carbonyl compounds present in liquid smoke contribute primarily to its antimicrobial activity. The amount of phenols present in liquid smoke condensates has been reported to be approximately 9.9-11.1 mg/mL (Ramakrishnan and Moeller, 2002). Phenolic compounds are known to disturb the cytoplasmic membranes of bacteria and cause the intracellular fluids to leak (Davidson, 1997). Carbonyls have been reported in liquid smokes in amounts of approximately 2.6 to 4.6% (Milly et al., 2005). Carbonyls inhibit microbial growth by penetrating the cell wall and inactivating enzymes located in the cytoplasm and the cytoplasmic membrane (Milly, 2003). Carbonyls act by condensing with the free, primary amino-groups in the polypeptide chains, primarily in the side chains of basic amino-acids. These amino groups may be an essential part of the active site of the enzyme, or they may function to bind the substrate by hydrogen-bonding (Painter, 1998). Even if the carbonyls cannot access the interior of a microbial cell, they can still inhibit growth by interfering with the uptake of nutrients. Table 1.6 shows some of the previous research studies that investigated liquid smoke as an antimicrobial in foods (Lingbeck et al., 2014).

Table 1.6. Liquid smoke evaluated as an antimicrobial in foods.

Bacteria/strain	**Liquid smoke concentration**	**Food substrate**	**Result**	**References**
Listeria monocytogenes Scott A, V7, 101 M	0.6% and 0.2%	Hot dog exudates	Listeria counts decreased after 3 days. Estimated D-values are 4.5 h at 0.6% and 36 h for 0.2%	Faith et al. (1992)

Listeria innocua	60%	Salmon	A 15 s dip resulted in a 3-log reduction. Greater reductions were seen with longer dip times	Vitt et al. (2001)
L. innocua ATCC 33090	0.9%	Salmon strips	Reduced *L. innocua* to 2 log CFU/g after 2 weeks	Montazeri et al. (2013)
L. monocytogenes CECT 932	100%, 1 min dip	Salmon filets	Fractions L1 and L2 immediately reduced Listeria to below detectable levels. Fraction S slowly reduced Listeria and was below detectable levels by 21 days. Fraction L3 did not show any inhibitory affects.	Sunen et al. (2003)
L. monocytogenes strains 4–121 and 1455	100%, 50%, 25%, 10% dipped	Salmon filets	Internal minimum lethality temperatures were >82.8 °C in untreated salmon steaks, 67.2 °C generated smoke was applied throughout the entire smoking process or >80 °C when smoke was only applied during the last half of the process, 58.9 °C when dipped in 100% CharSol C-10 and 62.8 °C, 68.9 °C and 72.8 °C with 50%, 25% and 10% liquid smoke.	Poysky et al. (1997)

L. monocytogenes Scott A-2, V7-2, 39-2, 383-2	100% dipped for 1 or 5 s or sprayed, 100% sprayed, 100%, 1 s dip	Frankfurters	Listeria was reduced to undetectable numbers after 4 weeks with all treatments	Gedela et al. (2007)a
L. monocytogenes ARS V67, ARS V72, ARS V113, ARS V125, ARS V105, LCDC 81–861	100% sprayed to equal 1.8 mL per frank	Frankfurters	Listeria was reduced by nearly 3 logs after 30 days and continued to slowly decline for up to 130 days	Martin et al. (2010)
L. monocytogenes LCDC 81–861, M1, M2, M5, C6, serotype 4b derived ATCC 19115	100%, dipped	Frankfurters	Listeria was reduced initially by at least 1 log and was undetectable after 72 h.	Messina et al. (1988)
L. monocytogenes	Formulated into franks at 10, 5, and 2.5% (w/w)	Frankfurters	Frankfurters formulated with 2.5% liquid smoke saw a 0.5 log CFU/mL reduction when inoculated at high Listeria levels and ~2 log CFU/mL when inoculated with lower levels of Listeria. Frankfurters formulated with 5% smoke saw greater reductions and were listericidal after 6 weeks. 10% smoke was listericidal after 4 weeks.	Morey et al. (2012)
L. monocytogenes Scott A-2, V7-2, 39-2, 383-2	100%, 1 s dip	Deli turkey	Listeria levels were reduced by 2 logs after 2 weeks and remained low after 10 weeks	Gedela et al. (2007)b
L. innocua M1	100%, 60 s dip	High end turkey rolls	Listeria levels were reduced to	Milly et al. (2008)

			undetectable levels after 2 and 4 weeks of storage on both high- and low-end turkey rolls with all smoke samples tested, with the exception of S4 on low end rolls. Only a small reduction was seen after two weeks (0.53 log CFU/g) but was not detected after 4 weeks.	
L. innocua M1	100%, 60 s dip	Roast beef cuts	Listeria levels were reduced to undetectable levels after 2 and 4 weeks of storage with all fractions tested.	Milly et al. (2008)
Escherichia coli O157:H7	8%	Beef trimmings	Growth was reduced by 2.3 log CFU/g	Estrada-Muñoz et al. (1998)
Staphylococcus aureus	25%, 50%, 75%, 100%	Brined strips	25% smoke treatment was ineffective on *S. aureus*. 50-100% smoke caused bacterial count reduction after 3-5 days.	Paranjpye et al. (2004)
Cocktail mixture of *S. aureus* ATCC 27664 (enterotoxin E), ATCC 13565 (enterotoxin A), and ATCC 12660	1.25%, 75%	Ground pork bellies	At 1.7 log reduction after 3 h compared to control. No reduction after 15 h compared to control, however no enterotoxins were present in smoke treated samples	Taormina and Bartholomew (2005)

To our knowledge, there have been a limited number of research studies that evaluated liquid smoke as an antimycotic agent in food substances. Efficacy of liquid smoke to inhibit mold growth in cellulosic meat casings had been evaluated by Chiu (1983) and against *Aspergillus* and *Penicillium* spp. in cheese had been evaluated by Wendorff et al. (1993). Milly et al. (2005) determined the minimum inhibitory concentration of liquid smoke against *Aspergillus niger* in nutrient broth. Similarly, there is limited knowledge on the evaluation of liquid smoke on stored food mite species including *T. putrescentiae.* Eischen et al. (2004) tested wood smokes from 40 different plants on *Varroa* mites (bee mites) and found that smoke from creosote bush and grapefruit leaves inhibited (60-100% mortality) or repelled the mites. Therefore, the antimycotic and acaricidal potential of liquid smoke in food substances warrants exploration.

Organic acid mixtures – Activate DA™ and Activate WD-MAX™

The organic acid mixtures Activate DA™ and Activate WD-MAX™ (Novus International, St. Louis, MO, USA) are nutritional feed acid products containing a blend of organic acids and methionine hydroxy analogue (2-hydroxy-4-(methylthio)butanoic acid, HMTBa), a bioavailable source of methionine. The combination of organic acids reduces the pH of the gastrointestinal tract of the animal, promotes the establishment of a balanced intestinal flora and aids in digestion, providing more nutrients from feed and improving animal performance. Activate DA nutritional feed acid is a combination of granular organic acids and HMTBa (total acid, 85%; HMTBa, 30%) that has been applied to premixes and finished animal feeds. Activate WD MAX (Acids, 50%; HMTBa, 33%) has been used in drinking water for animals, providing nutritional benefits (methionine activity) and drinking water acidification, making the drinking water a less favorable environment for pathogen growth (e.g., *Salmonella, E. coli, Campylobacter*, etc.).

Uses of HMTBa as a feed supplement and an antimicrobial

The amino acid methionine is identified as a limiting amino acid in high forage cattle diets and has a positive impact on the health and performance of the animal. Methionine participates in a wide variety of metabolic pathways and serves as a precursor to other amino acids, like cysteine. Methionine is not synthesized within the animal's tissues; therefore, it must be present in the diet or provided by the rumen microbes. However, it is difficult for growing beef cattle to get enough metabolizable, or bypass, methionine in their diets. Because methionine plays several roles in an animal's metabolism and it cannot produce this essential amino acid on its own, it is important to supplement the diet. Adding a high-quality methionine supplement has been shown to support oxidative balance and improve feed efficiency, proper metabolic activity and lean growth, which all lead to improved performance, better reproduction and increased profits. If free amino acids are fed directly to beef cattle, the rumen microbes destroy them before they even leave the rumen. Because amino acids must be presented to the small intestine in required amounts for the animal to synthesize protein, the amino acid must be protected or modified to avoid rumen degradation. Supplementing rations with a methionine hydroxy analogue is an economical way to supply methionine and have a positive impact on the health and performance of the animal. HMTBa (2-hyroxy 4-methlythio butanoic acid) being an organic acid and a methionine hydroxy analogue has been used as a methionine precursor in animal feed due to its unique chemical structure that allows protection from some of the microbial degradation in the rumen gut. HMTBa also provides acidifying effects of organic acids which subsequently provide gut health advantages to the animal by mitigating pathogen growth in the gut (Kaewtapee et al., 2010; Swennen et al., 2011). Methionine hydroxy analogue has also been shown to reduce nitrogen excretions (Kim et al., 2014), support animal performance during heat stress (Knight and Dibner, 1984; Dibner et al.,

1992) and offer antioxidant capacity (Feng et al., 2011; Willemsen et al., 2011; Kuang et al., 2012; Li et al., 2014).

There have been only a few studies that investigated the antimicrobial properties of organic acid mixtures including HMTBa. Guo-zheng et al. (2012) evaluated Activate WD against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella pullorum*, and *Campylobacter jejuni* and determined the minimum inhibitory concentration to be 0.3% and minimum bactericidal concentration to be 0.6%. Parker et al. (2007) evaluated Activate WD at 0.04 and 0.08% in drinking water for poultry and found reduction in horizontal transmission of *Salmonella* in the broiler chickens. These studies show that the organic acid mixtures containing HMTBa have potential antimicrobial effects in feed.

Conclusion

There is increasing interest in the use of natural, GRAS-certified, clean label ingredients in pet food as an alternative to chemical preservatives. Liquid smoke is an effective antimicrobial against an array of bacterial pathogens as demonstrated in both broth culture and food systems. Commercial use of liquid smoke in the food industry may satisfy consumer demand for all-natural foods while still maintaining their safety. Liquid smoke is being used more frequently in preserving protein-based foods, namely meat, fish, and cheese, because it imparts a pleasant flavor and also has inhibitory effects on food borne pathogens. The preservative effect of liquid smoke is achieved by antimicrobial and antioxidant compounds such as aldehydes, carboxylic acids and phenols. Liquid smoke also has several advantages over traditional smoking techniques including ease of application, speed of smoking process, good reproducibility of desired characteristics obtained in the final smoked food, and omission of hazardous polycyclic aromatic hydrocarbons. Evaluating its antifungal and acaricidal potential can help diversify its use in protecting intermediate moisture

foods like semi-moist pet food from mold contamination and mite infestation. Similarly, the use of methionine hydroxy analogues and organic acid mixtures have been so far as animal feed supplements and acidification of drinking water. Investigating their potential as an antimicrobial and antifungal agent in low moisture foods like pet food kibbles and as food-grade sanitizers on food contact surfaces can diversify their application in high value food products (human and pet foods) and safe, non-toxic addition to conventional sanitizing of food processing spaces.

Hypotheses

Considering the antimicrobial and preservation properties of liquid smoke, we hypothesized that liquid smoke preparations may have acaricidal and antimycotic properties to control mite and mold growth respectively, in semi-moist pet food. Further, due to the reason that organic acids like HMTBa have acidification and pH-lowering properties we hypothesized that organic acid mixtures containing HMTBa when used as a coating on pet food kibbles and on pet food contact surfaces can mitigate pathogenic bacteria and molds during post-processing contamination.

Objectives

The objectives of the research studies presented in this dissertation were:

(i) To determine the effects of liquid smoke preparations on the storage mite, *Tyrophagus putrescentiae,* population growth and orientation (attraction/aversion) behavior in a model semi-moist pet food system.

(ii) To investigate the effects of liquid smoke preparations on storage fungi, *Aspergillus flavus* and *Fusarium graminearum,* by determining their minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) in nutrient broth.

(iii) To determine the number of days to observe wild-type mold growth in a model semi-moist pet food prepared with liquid smoke inclusions at various concentrations.

(iv) To evaluate the effects of liquid smoke preparations on the growth of *Aspergillus flavus* using a mold-challenge study in a model semi-moist pet food system.

(v) To determine the efficacy of organic acid mixtures, Activate DA and Activate WD-MAX coated on extruded dry pet food kibble on the survival of *Salmonella enterica*, *Escherichia coli* and *Aspergillus flavus* using challenge studies.

(vi) To evaluate the residual antimicrobial effect of organic acid mixtures, Activate DA and Activate WD-MAX coated on extruded dry pet food kibble on *Salmonella enterica*, to maintain *Salmonella*-free kibble despite repeated exposure over time.

(vii) To determine the efficacy of organic acid mixtures, Activate DA and Activate WD-MAX applied on pet food contact surfaces on the survival of *Salmonella enterica*.

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Chapter 2 - Efficacy of liquid smoke preparations to mitigate infestation of the storage mite, *Tyrophagus putrescentiae*, in a model semi-moist pet food system

Abstract

The storage mite, *Tyrophagus putrescentiae*, infests a wide range of food products including pet food. Control of this mite depends on chemical methods such as fumigation and spraying with insecticides. Methyl bromide was used as a fumigant for stored products, especially to control storage mite infestation, but it is now banned for most uses in many countries because of its ozone-depleting effects. Effective alternatives to methyl bromide are needed to develop integrated pest management programs for this pest. Liquid smoke is a naturally derived flavorant and preservative with known antimicrobial properties. The objective of this study was to investigate the efficacy of eight liquid smoke preparations with varying phenol and carbonyl concentrations and pH, on *T. putrescentiae* survivability and orientation behavior in a model semi-moist pet food. Survivability of *T. putrescentiae* on treated (0.3% smoke inclusion) and untreated (0% smoke) semi-moist pet food samples was determined by enumerating their population at 7, 14, and 28 days post-infestation. Untreated semi-moist pet food cubes were dipped in liquid smoke preparations at 0%, 0.3%, 1%, 5%, 10%, 25%, 50% or 100% concentration, and used in two-choice behavioral assays. The attraction or repulsion of mites towards the treatments was determined by enumerating mites on samples at 2, 8, and 24 h, and a repellency index (RI) was calculated. At 14 days, the mean mite population among smoke treatments ranged from 207-244 when compared with the untreated (212 mites), the positive control (propylene glycol treatment; no mite growth) and the negative control (mite rearing diet; 454 mites). Among the smoke treatments, Cloud S-C100, Code-10 and Cloud S-AC15 repelled mites the most (-10% to +60% RI). There was no difference among treatments for survivability; therefore, liquid smoke did not kill or inhibit the mite population growth. However, some liquid smoke fractions may provide repellency to retard mite infestation in stored semi-moist foods.

Key words: *Tyrophagus putrescentiae*, Liquid smoke, Mold mite, Semi-moist pet food

Introduction

One of the most important sources of food allergens worldwide are stored food mites (Vogel et al., 2015), mainly *Tyrophagus putrescentiae*. This stored food mite is a cosmopolitan species frequently found in a wide variety of stored products. It is commonly known as cheese or mold mite and is also sometimes referred to as ham mite. It is particularly common in foods with high levels of fat and protein, such as dried eggs, ham, fish meal, cheese, nuts and pet foods (Rodriguez et al., 1987; Chambers, 2002). It is a significant cause of allergic asthma and allergic rhinitis among grain handlers, bakers, and food industry workers because it occurs in large numbers in farm buildings and in food manufacturing and storage facilities. It is also being recognized as an important contributor to the allergen content in dust in homes (Iversen et al., 1990). Furthermore, the stored food mites are responsible for acute enteritis and severe systemic reactions or anaphylaxis, as a result of the consumption of food that was infested with these mites (Matsumoto et al., 1996). Mites are also implicated in the transmission of pathogenic micro-organisms, mold spores, and prions (Griffiths et al., 1959; Wisniewski et al., 1996).

Stored product mites may be considered important allergens in dogs causing atopic dermatitis. In a recent study (Thind, 2005), three species of storage mites including *T. putrescentiae*, were captured from the processing and packing areas of a pet food manufacturing factory in the United Kingdom, even in the cleanest locations. This suggests that pet food ingredients or even the finished products could become a suitable source of stored product mites. Commonly, the stored product mites live on the external surface of these food products, but sometimes they penetrate inside, causing serious economic losses (Zdarkova, 1991). When pet foods are stored at home by the consumers, they are susceptible to mite infestation from house dusts which may harbor some of the storage mites. Brazis et al. (2008) observed that two out of

ten different brands of sealed commercial dog foods contained storage mites, and upon storing those at an optimal temperature and humidity, nine out of ten of them contained storage mites.

Traditionally, control of mites depends on chemical methods such as fumigation with methyl bromide, spraying cracks and crevices with organophosphorus compounds, or treatment with pesticides like benzyl benzoate and repellents like DEET (N,N-diethyl-m-toluamide) (Collins, 2006). Residual pesticides are mainly used to treat the structure of buildings, and some are applied directly to the food commodity. Methyl bromide, though an effective fumigant used to control storage mites like *T. putrescentiae* has been phased out in industrialized nations across the world including the United States due to its ozone-depleting nature (EPA, 2006). Numerous efficacy tests have been performed with experimental and formulated acaricides against mold mites in the context of agricultural and industrial settings (Kim et al. 2003; Collins et al., 2006; Palyvos et al., 2006; Hubert et al., 2007; Jeong et al., 2008; Stara et al., 2011). They leave behind some amount of residue on the treated commodity, and repeated use of these chemicals have resulted in the development of resistance in mites (Szlendak et al., 2000). These chemicals have been shown to cause undesirable effects on non-target organisms and have fostered environmental and human health concerns (Hays et al., 1991). These problems have highlighted the need for the development of new strategies for selective storage mite control, preferably using safe, non-toxic, and natural compounds.

To date there are no targeted intervention strategies against storage mites that pet food industries could follow beyond the use of propylene glycol as a humectant. However, this polyol is not a preferred ingredient by many consumers and is poorly tolerated by cats as it causes Heinz body formation in their blood leading to anemia (Weiss et al., 1990). Furthermore, some consumers have considered propylene glycol as a controversial additive to use in food products

mainly due to its similarity to the toxic ethylene glycol, which is a key component in anti-freeze (Dog Food Advisor, 2009). Replacing propylene glycol with glycerol, which is not toxic to cats, is a preferred ingredient option. However, this creates an opportunity for mite growth or infestation (Abbar et al., 2016). Therefore, a strategy to use an alternative, such as liquid smoke, might be a new solution.

Liquid smoke is a naturally derived flavor component and preservative used in human and pet foods, with known antimicrobial properties. Liquid smoke is approved by the US-FDA as a "Generally Recognized as Safe" (GRAS) substance and can be included in human and pet foods with no specific limit, and the flavor of liquid smoke is widely likeable in these foods as well. There is no available information on its acaricidal potential against storage mites. Eischen et al. (2004) tested wood smokes from 40 different plants on *Varroa* mites (bee mites) and found two of them to be effective. To our knowledge, there are no studies that tested liquid smoke on the stored product mite *T. putrescentiae.* Ernst et al. (2007) reported that coating pet food with conjugated linoleic acid inhibited *T. putrescentiae* growth and infestation. Abbar et al. (2016) evaluated *T. putrescentiae* population growth and orientation behavior on dry cured ham pieces with and without coating by various food safe components like propylene glycol and butylated hydroxytoluene. Manu et al. (2021) reported that a mixture of three short-chain fatty acids $C_{8,}$ $C_{9,}$ C_{10}, and the sesquiterpene ketone nootkatone coated on ham cubes repelled *T. putrescentiae*. Other than these, we could not find any published literature that tested inhibition or repellency effect of food-safe components on *T. putrescentiae* infestation. The objective of this study was to investigate the effects of liquid smoke preparations on the storage mite *T. putrescentiae* population growth and orientation (attraction/aversion) behavior in a model semi-moist pet food system.

Materials and Methods

Preparation of semi-moist pet food and source of liquid smoke

The formula used for the preparation of semi-moist pet food is shown in Table 2.1. All the ingredients were weighed according to the formula (Table 2.1), to produce 2 kg of pet food per batch. A 3.3 L planetary mixer (KitchenAid Portable Appliances, St. Joseph, MI, USA) was used to mix the ingredients at a speed of about 50 rpm for 10 min. The dry ingredients were mixed first, followed by the liquid ingredients. The mixture was spread in a uniform layer of approximately 1 cm thickness on a baking tray lined with parchment paper. It was baked in a convection oven (MEA 21-93-E; Garland Commercial Industries, PA, USA) at 175°C for 10 min. The baked pet food was cooled on a wire rack until it reached room temperature. It was then cut into uniform cubes of size 3×3×1 cm using a stainless-steel knife.

Table 2.2 is a list of eight liquid smoke preparations, S1 to S8. These were provided by Kerry Inc. (Beloit, WI, USA).

Mite culture

Laboratory cultures of *T. putrescentiae* have been maintained in the Department of Entomology at Kansas State University for more than 4 years and have not been subjected to any pesticides. The mites were reared in glass jars containing mite diet similar to the culture used in the study by Abbar et al. (2016). The glass jars were sealed with labeled filter paper in the metal lid ring. The mite rearing diet was composed of agar, yeast, cellulose (Alphacel, ICN Biomedicals, Costa Mesa, CA, USA), mixed vitamins (Vanderzant modification vitamin mixture for insect diet; MP Biomedicals LLC, Irvine, CA, USA) (5:5:5:5 g), dog food (Purina Beneful, Nestlé-Purina Pet Care, St. Louis, MO, USA) (160 g), glycerol, antifungal salt solution in ethanol (methyl-p-hydroxybenzoate, 15:85 g mL^{-1}) and water (25:25:475 mL), which were mixed and cooked for

about half an hour and then added to the dog food in rearing jars. Mites from the already maintained laboratory culture were introduced to new cultures after the diets had been cooled to 25°C. The jars were stored in an incubator at 25°C and 70% RH in total darkness.

Treatments used in the study

For the mite population growth study (Experiment 1), the liquid smoke preparations P-1720 (S1), Cloud S-5 (S2) or Cloud S-C100 (S3) (Table 2.2) were evaluated at 0.3% (w/w) inclusion in semi-moist pet food. Semi-moist pet food with no added smoke served as the untreated control. A standard mite rearing diet (not a semi-moist pet food product, and no added smoke) served as the negative control. Semi-moist pet food dipped in 20% propylene glycol solution for 2 min and air dried for 1 min served as the positive control. Three replications were done for this study.

For the mite orientation behavior study (Experiment 2), all the liquid smoke preparations S1, S2, S3, S4, S5, S6, S7 or S8 were evaluated at concentrations 0.3%, 1%, 5%, 10%, 25%, 50%, or 100% v/v (by volume in water) as coating on semi-moist pet food for sampling time periods 2, 8, and 24 h, in comparison to untreated control (0% smoke). The coating was done by dipping the semi-moist food cubes in liquid smoke at the desired concentration levels for 2 min and air dried for 1 min. A propylene glycol treatment (positive control) was evaluated at 20% concentration as coating on the pet food for 2, 8, and 24 h. Each treatment combination was replicated three times in a completely random design.

Experiment 1: Mite population growth assay

A standard mite diet (Abbar et al., 2016) was composed of agar, yeast, cellulose, pre-mixed vitamins at a ratio of 20:5:5:5 g, glycerol, antifungal salt solution in ethanol (methyl-p-hydroxybenzoate, 15:85 g mL^{-1}) and water (25:25:475 mL), mixed and cooled to set, and cut into

3×3×1 cm cubes. The standard mite diet was included to determine the reproductive potential of *T. putrescentiae*. The untreated and smoke treated (0.3% of P-1720, Cloud S-5 or Cloud S-C100) semi-moist pet food cubes, and the mite diet cubes were placed separately in glass jars (216 mL; Ball Corp., Broomfield, CO, USA) into which 20 adult mites were introduced. For the positive control of a compound known to inhibit mite reproduction, the untreated pet food cubes were dipped in 20% propylene glycol for 2 min, air dried for 1 min and then placed in the jars. The jars were sealed with labeled filter paper under the jar ring after mite inoculation. To prevent the mites from leaving the jars, the inside rim of the jars was smeared with petroleum jelly. The sealed jars were placed in plastic tubs containing detergent solution to reduce the surface tension of water so that the mites were prevented from escaping. The jars were stored in an incubator at 25°C and 70% RH in total darkness. The number of live mobile adult mites in the jars were counted after 7, 14, and 28 days later. Each treatment combination was replicated three times.

Experiment 2: Mite orientation behavior assay

Two-choice behavioral assays described by Abbar et al. (2016) were conducted with mites to determine whether their orientation towards semi-moist pet food was affected by treatment with liquid smoke. Small cubes of semi-moist pet food (5×5×5 mm) were used in the two-choice tests. The cubes were placed in bioassay arenas (Figure 2.1) which consisted of 90×20 mm glass Petri dishes that had a 90 mm diameter circular piece of black construction paper covering the floor of each dish on the underside. Three circles of 18 mm diameter were centered along a line passing through the center of the paper, with one circle in the middle of the floor and one circle each at a distance of 5 mm from the side wall. For this study, one ‘treated’ pet food cube and one ‘control’ pet food cube, each 5×5×5 mm was placed in each of the circles, B and C. Twenty adult mites of mixed sex were released into the middle circle A (Figure 2.1). The arenas were then placed in total

darkness at 25°C and 70% RH. Mites were prevented from leaving bioassay dishes by applying a thin layer of vacuum grease along the inside upper 5 mm of the Petri dish bottom. For treated samples, pet food cubes were dipped in 20% propylene glycol or 0.3%, 1%, 5%, 10%, 25%, 50%, or 100% (v/v) of the liquid smoke preparations S1, S2, S3, S4, S5, S6, S7 or S8 respectively for 2 min. In the control treatment, untreated pet food cubes were dipped in sterile distilled water (0% smoke) for 2 min. The pet food cubes were then air dried for 1 min before placing in the bioassay arena. Mites that oriented towards each of the pet food cubes within the circles or those on the food surface were counted after 2, 8, and 24 h, and these experiments were replicated three times.

The attraction or repulsion of the mites towards smoke treatments was calculated by a repellency index (RI) and expressed as a percentage, according to Manu et al. (2021) using the following equation:

$$RI = (N_c\text{-}N_t)/T \times 100$$

where, N_c = number of mites on the control pet food; N_t = number of mites on the liquid smoke treated pet food; T = total number of mites released (T=20).

Positive values of RI indicated 'repulsion' and negative values of RI indicated 'attraction'.

Statistical analysis

For the population growth assay experiment, data were subjected to two-way analysis of variance (ANOVA) and means among treatments were separated using Tukey's post-hoc test when the *F*-test of the ANOVA per treatment was significant at $P<0.05$. For the orientation behavioral assay experiments, data were subjected to three-way analysis of variance (ANOVA), followed by two-way ANOVA at each time period. Means among treatments at the 24 h time period were separated using Tukey's post-hoc test when the *F*-test of the ANOVA per treatment was significant

at $P<0.05$. All analyses were conducted using the statistical software SAS version 9.3 (SAS Institute, 2011).

Results

The mite reproduction assays indicated that (Table 2.3 and Fig 2.2) at 7 days the mean mite population on the smoke-treated (P-1720, Cloud S-5 and Cloud S-C100) semi-moist pet food samples ranged from 89 to 95, and that on the untreated pet food was 90 mites. The mite culture diet had a mite population of 316 at 7 days. At 14 days, the average mite population among smoke treatments ranged from 207 to 244 which was similar to the untreated (212 mites) semi-moist pet food, but less than that observed on the mite rearing diet (454 mites). At 28 days, the mite population among the untreated and smoke treated pet food samples were too numerous to count (data not shown). In the propylene glycol treatment (20% propylene glycol, positive control), no mite growth was observed. From the mite population counts in the treatment samples and grouping of treatment means by Tukey's test (Table 2.3; $F = 64.91$; df = 5, 24; $P<0.05$), it was concluded that none of the liquid smoke preparations killed the mites, significantly inhibited their reproduction, or limited their population growth.

For the two-choice behavioral assays (Table 2.4 and Fig 2.3), at 5%, 10%, 50%, and 100% smoke concentration the mean mite repellency index was the highest (20-60% RI) for Cloud S-C100 and Cloud AC-15. At 1% and 0.3% smoke concentration the repellency index was the highest (10-20% RI) for Cloud S-5 and Code V among all time periods. At 25%, 50%, and 100% smoke concentration P-1720, Hickory OS-1473 and Code 10 attracted mites the most (-30 to -40% RI). At 10% smoke concentration Cloud S-5, Black deli and Code V attracted the mites the most (-50% RI) among all time periods. At 5% and 1%, Hickory OS-1473 and Black deli attracted the mites the most (-40 to -50% RI) respectively, while at 0.3% Code 10 attracted mites the most with

an RI of -40%. From Table 2.4, grouping of treatment means by Tukey's test at 24 h time period (F = 4.15; df = 14, 41; P<0.05) indicated that, across different concentrations of liquid smoke treatments Cloud S-C100 and Cloud S-AC15 containing medium to high carbonyl content were the most effective at repelling the mites followed by Cloud S-5 (low carbonyl, no phenol) and Code V (medium carbonyl, low phenol) smoke preparations. The least effective treatments to repel mites were P-1720, Black deli, Hickory OS-1473 and Code 10.

Discussion

The mold mite *T. putrescentiae* readily infests foods having a high fat or protein content and moisture content in the range of 12 to 18% (Erban et al., 2015). Therefore, the semi-moist pet food is a highly suitable model food substrate for investigating acaricidal properties of natural food preservative components like liquid smoke. The results of this study provide information regarding relationship between the effects of various liquid smoke treatments on mite population growth and their attraction or aversion behavior towards the liquid smoke preparations.

Results from the population growth experiments demonstrated that the three liquid smoke preparations tested in semi-moist pet food at 0.3% inclusion w/w, namely Cloud S-5, P-1720 and Cloud S-C100, did not reduce or inhibit the population growth of mites when compared to the untreated (no smoke) pet food. These liquid smoke preparations had carbonyl content ranging from low and medium to high. These were incorporated in the semi-moist pet food at 0.3% by weight, as it was the inclusion level recommended by the supplier based on acceptability by pets (dogs) and feasibility. The number of mites on the smoke treated and untreated pet food samples by day 7 was in the range of 80 to 100 from an initial inoculation of 20 mites, and by day 14 the mite counts were in the range of 200 to 300. By day 28 the mites were innumerable to count. The population doubling time for *T. putrescentiae* under optimum growth conditions of 30°C and 90%

RH is about 1.75 days (Sanchez-Ramoz et al., 2005). Accordingly, mite growth in our study at 25°C and 70% RH on the mite culturing diet was about 300 mites on day 7 and 450 mites on day 14, which was a little lower, possibly due to the lower temperature and relative humidity conditions. However, the mite rearing diet had a higher moisture content of 70% compared to the semi-moist pet food which had 26% moisture content. This explains the slightly lower mite population growth on the semi-moist pet food samples compared to the mite diet, even though the temperature and relative humidity conditions were the same for both. Our mite count results were similar to the results obtained by Abbar et al. (2016), who tested mite population growth on dry cured ham pieces with and without coating by various food safe components. Their control treatment which was untreated dry cured ham pieces coated with water had similar counts (200-300 mites) at the end of two weeks. We believe that the 0.3% inclusion level of smoke in the semi-moist pet food could be too low of a concentration level to cause any inhibitory or killing effect on mite population growth. However, incorporating very high concentrations of smoke in pet food is impractical and not feasible due to the strong flavor and aroma which could be unpalatable for the pets.

As it was determined from the population growth assay that these liquid smoke treatments at an inclusion level of 0.3% in semi-moist pet food did not inhibit mite growth, the next experiment was designed to determine the attraction or repellency effect of the various liquid smoke preparations at different concentration levels on a wider range (0.3% to 100%) using two-choice behavioral assays. For the behavioral assays the smoke treatments S1 to S8 were tested as coatings on the semi-moist pet food, as it enabled testing a wide range of concentrations (0.3%, 1%, 5%, 10%, 25%, 50%, and 100% v/v of water) in a shorter time frame when compared to incorporating all these eight smoke preparations at these seven concentration levels in the recipe.

Coating of smoke treatments on semi-moist pet food can also be a feasible approach to apply higher concentrations and test their attraction or repellency effect on mites, as it is surface application and not incorporated within the recipe. Surface application means less masking or buffering effect of the smoke components by the other ingredients in the pet food recipe, and at the same time can also have a higher concentration effect as it is applied only to the surface of the pet food sample.

Measuring the activity of a repellent on insects and mites using behavioral tests have been reported by researchers (Birkett et al., 2008, 2011; Nechita et al., 2015; Kim et al., 2018; Rajabpour et al., 2018; El Adouzi et al., 2019; Lee et al., 2019). These researchers studied the repellency effects of volatile organic compounds and essential oils of plant origin on different species of mites using different types of choice-behavioral test strategies namely Y-tubes (Bock and Cardew, 2008), T-tubes (El Adouzi et al., 2019), four-way olfactometers (Bruce et al., 2015; Deletre et al., 2016), two pieces of repellent-impregnated filter paper in a Petri dish (Rajabpour et al., 2018), or cardboard traps impregnated with the repellent chemical (Birkett et al., 2011). Repellency effects of liquid smoke preparations have been investigated for the first time in this study. We used two-choice behavioral assays similar to the study by Abbar et al. (2016) using the liquid smoke-impregnated/coated food sample on one side and the control food sample on the other side in a glass Petri dish (bio-assay arena). Results from the behavioral studies indicated that mites avoided semi-moist pet food samples coated with liquid smoke preparations Cloud S-C100 and Cloud S-AC15 for the majority of concentrations tested (5%, 10%, 50% and 100%). These smoke preparations had medium to high carbonyl content, some organic acid and low phenol content. Cloud S-5 (low carbonyl, no phenol) and Code V (medium carbonyl, low phenol) smoke preparations also repelled the mites at low concentrations (1% and 0.3%). It was surprising that

these two liquid smokes did not repel mites at some of the higher concentrations (10% and 25%) tested. The reason could be that the mites were introduced in the middle region of the bioassay arena, with the control and smoke treated pet food samples on either side. We speculate that when suddenly confronted with the volatile, attraction/repulsion-inducing compounds present in liquid smoke, the mites started moving away from both the control and smoke treated samples and wandered off outside the test region (the three circles A, B, and C of 18 mm diameter) of the arena. This is a disadvantage of the two-choice bio-assay arena, because it provided enough empty space for the mites to wander off away from the test area where the food samples were kept. To overcome this disadvantage to some extent, we enumerated the mite counts on and in the circles of the control and smoke treated samples at three different time intervals at 2 h, 8 h and 24 h. This would give the mites a longer time (24 h) to get accustomed to the volatile compounds in the arena and make a choice and move towards their target food sample. The smoke preparations P-1720, Black deli, Hickory OS-1473 and Code 10 were the least effective at repelling the mites with negative repellency indices. It was also surprising that some liquid smokes like Cloud S-5 and Code V had the highest repellency indices at some of the concentration levels (1% and 0.3%), but also attracted the mites at another concentration level (10%). These results suggest that the intensity of repellency (or attraction) can actually depend on the number and physiological status of mites used in the behavioral assays, the concentration and ratio of various compounds present in the liquid smoke preparation, and temperature and humidity conditions (Visser, 1988; Deletre et al., 2016; Lee et al., 2019). Visser (1988) reported that starved and satiated phytophagous insects did not behave similarly in terms of seeking host plants. We believe that the satiety of mites we used from the laboratory culture can influence their movement towards or away from the test food samples used in our behavioral study. Also, we introduced 20 adult mites in the assays and so the repellency

indices we obtained were based on this limited number of mites' movement in a span of 24 h. Deletre et al. (2016) suggests that the effect induced by a compound depends on its concentration and the duration of exposure to mites. Lee et al. (2019) studied the repellency effects of cinnamon oil, clove oil and their volatile organic compounds depending on the evaporation time against poultry red mites. They showed that both the essential oils were repellent regardless of evaporation time, however, two components of clove oil namely eugenol and eugenol acetate were found to change from having a repellent to an attractant effect over time in the experiment. They proposed that this may be partly explained by a difference in the dynamics of evaporation between the single compounds and the whole essential oil. Liquid smoke also contains several components from wood/plants like phenols, carbonyls and organic acids along with volatile compounds that may have different evaporation dynamics thus changing their effects over time. In spite of these, the smoke preparations Cloud S-C100 and Cloud S-AC15, followed by Cloud S-5 and Code V, had quite high repellency indices (up to 60% RI) at most of the concentrations tested which enables their use as repellents to some extent in semi moist foods.

Plants have been used for centuries in the form of crude fumigants where plants were burnt to drive away mosquitoes. Plants have repelling constituents like alkaloids, terpenoids and phenolics that target predators that attack them, and wood smokes are known to contain these compounds as well. Baltes et al. (1981) found the major proportion of commercial full-strength liquid smoke to be composed of water (11-92%), tar (1-17%), acids (2.8-9.5%), carbonyl containing compounds (2.6-4.6%) and phenol derivatives (0.2-2.9%). However, in the manufacturing of liquid smokes, a variety of ingredients may be used, such as salts, fatty acids, fatty esters and carriers like saccharides (Guillen et al. 1997; Varlet et al. 2010). Phenolic compounds contribute to smoke flavor and color of liquid smokes, and also have antibacterial and

antioxidant properties (Clifford et al. 1980; Maga 1987; Varlet et al. 2010). Carbonyl-containing compounds impart sweet or burnt-sweet aroma and tend to soften the heavy smoky aroma associated with phenolic compounds with some 'typical smoke-cured' aroma and flavors (Fujimaki et al. 1974; Kim et al., 1974; Kostyra et al., 2006). Wood smokes, phenolic compounds in plant essential oils, and fatty acids were found to repel or inhibit growth of mites by several researchers. Eischen (1997) tested the efficacy of smoke from various plant materials, including gobernadora, eucalyptus, coffee beans, corncobs, pine needles, and tobacco on *Varroa* mites (bee mites) and found that smoke from creosote bush and grapefruit leaves caused 60 to 100% mortality or repelled the mites. Masoumi et al. (2016) studied repellent effects of carvacrol and thymol, the essential oils found in oregano and thyme, against mites. Carvacrol is a monoterpenoid phenol and thymol a monoterpene, both known for their acaricidal properties (Piccaglia et al., 1993; McGimpsey et al., 1994; Ahn et al., 1998; Porte et al., 2008). Ernst et al. (2007) reported inhibition of *T. putrescentiae* growth in pet food when treated with conjugated linoleic acid. Smoke is also known to contain terpenoids, phenolics and fatty acids. We hypothesize that the ratio and composition of carbonyls to phenols to volatile organic compounds in liquid smoke might be responsible for the repellency effect on mites in our behavioral assays.

The mechanism of attraction or repulsion towards chemicals by mites has been described by Carr and Roe (2016). Mites use chemosensory sensilla present on their palps and tarsi to identify olfactory and gustatory chemicals in the environment. Olfactory sensilla detect volatilized chemicals while gustatory sensilla detect chemicals through direct contact (Leonovich et al., 2011). It is hypothesized that the molecular mechanism involved in the binding of chemical molecules and neuron depolarization in olfactory and gustatory receptor cells share a similar signal cascade mechanism, though the exact proteins involved are still unknown (Carr and Roe, 2016). We

propose the repellency effect on the mites from the phenolic and other volatile compounds present in liquid smoke happens according to the two-step mechanism described by Deletre et al. (2016) for chemical/attractant response. The first step is the "choice" of host or favorable food substrate and consists of searching for and recognizing the food by means of olfactory and/or visual clues/stimuli. This choice is made at a distance from the food source. The second step is the 'selection' of the host/food, which consists of contacting and accepting the food and, in some cases, selecting a suitable feeding area on the food surface on the basis of contact chemoreception (or 'taste'). The repellency or attraction takes place through stimuli of receptors in the mites' body and can be influenced by the chemical/volatile compounds present in liquid smoke.

T. putrescentiae is also called mold mite as the presence of mold on food substrates help proliferate this mite species as it feeds on the mold. The research study presented in chapter 3 of this dissertation reports that liquid smoke preparations Cloud S-C100 and Cloud S-AC15 containing medium to high phenols had a fungistatic and fungicidal effect on the growth of the storage mold *A. flavus* in semi-moist pet food at concentration levels of 1 to 2%. This mold-inhibiting potential of liquid smoke can be an added advantage to deter mite infestation in semi-moist foods with the inclusion of liquid smoke. For future work, the appropriate concentration levels of inclusion of liquid smoke in semi-moist pet food needs to be determined through palatability tests with pet animals for practical application. A modified two-choice behavioral assay using T-tubes can be conducted which is more suitable for mite repellency tests, and an in-depth analysis of individual components in liquid smoke that can cause repellent effect also needs to be investigated to better understand the effects of liquid smoke on mites. Characterization of the components of liquid smoke would also help in exploring the use of combinations of smoke preparations to study their antagonistic and synergistic effects against target pests.

Conclusion

Methyl bromide has been an effective fumigant to control *T. putrescentiae* and other storage mite infestation in foods (Sekhon et al., 2010). However, it has been banned in several countries including the US due to its ozone-depleting nature. DEET is also one of the most commonly used active ingredient in insect and mite repellents (Soulie et al., 2021). As the use of such insecticides and fumigants is less safe in spaces like retail food stores and consumers' homes where food is stored, it is more practical and safer to use food-safe components like liquid smoke in semi-moist pet foods. In this study, liquid smoke did not kill or inhibit the mite population growth; therefore, it will not replace chemical fumigants at this time. However, the liquid smoke preparations Cloud S-C100, Cloud S-AC15 which contained medium to high carbonyl concentration and low phenols may provide some repellency effect and retard mite infestation in stored semi-moist pet food, when evaluated as coatings on semi-moist pet food. We propose that the use of these liquid smoke preparations in semi-moist foods along with good manufacturing practices (GMPs) and sanitation practices by the food industries would help in developing an effective integrated pest management program to control storage mite infestation.

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Tables & Figures

Table 2.1. Formula used for manufacturing the model semi-moist pet food.

Ingredient	Percent % (w/w)
Water	18.0
Corn	12.2
Chicken by-product meal	11.4
Corn gluten meal	11.4
Glycerin	12.2
Wheat	5.8
Choice white grease	5.8
Corn syrup	5.0
Gelatin	5.0
Rice flour	4.3
Soybean meal	4.3
Molasses	1.0
Dry dog digest	0.5
Salt	0.5
Dicalcium phosphate	1.4
Vitamin premix	0.2
Phosphoric acid	0.2
Potassium chloride	0.2
Lysine	0.1
Trace mineral premix	0.1
Calcium carbonate	0.1
Choline	0.1
Potassium sorbate	0.1
Natural antioxidant	0.1
Total	100.0

Table 2.2. Liquid smoke preparations evaluated in the study.

Liquid smoke preparation	Name	Description
S1	P-1720	Buffered low phenol smoke, medium carbonyl
S2	Cloud S-5	Buffered pH, low acid, low carbonyl, no phenol
S3	Cloud S-C100	Carbonyl preparation: high carbonyl, low acid, very low phenol
S4	Black deli	Basic pH smoke, zero carbonyls, organic acid salts, phenols
S5	Hickory OS-1473	Phenol preparation: high phenol, low acid, no carbonyl
S6	Code 10	Base smoke: organic acid/carbonyls/phenols
S7	Code V	Organic acid preparation: low pH, medium acid, medium carbonyl, low phenol
S8	Cloud S-AC15	High buffered organic acid + medium carbonyl preparation

Table 2.3 Mean mite population growth at 7 and 14 days on semi-moist pet food treatments with inclusion of liquid smoke preparations namely P-1720, Cloud S-5 and Cloud S-C100 at 0.3% w/w, in comparison to the untreated control (semi-moist pet food with no smoke added), and the mite rearing diet (lab culture diet). Mite population at 28 days was innumerable to count (data not shown). Propylene glycol treatment (positive control) did not show mite population growth.

Treatment	**Mite population (Mean ± SE)[abc]**	
	Day 7	**Day 14**
Mite diet	316.7 ± 32.1 $_{a, A}$	454.0 ± 50.5 $_{b, A}$
Untreated	90.3 ± 27.2 $_{a, B}$	212.0 ± 59.9 $_{b, B}$
P-1720	95.0 ± 30.4 $_{a, B}$	244.0 ± 32.4 $_{b, B}$
Cloud S-5	89.3 ± 31.9 $_{a, B}$	207.7 ± 34.2 $_{b, B}$
Cloud S-C100	91.7 ± 23.2 $_{a, B}$	216.7 ± 32.0 $_{b, B}$

[a]Each mean is based on $n = 3$ replications.

[b]Means among treatments across day 7 and day 14 followed by different letters in lower case are significantly different ($P<0.05$, Tukey's test).

[c]Means among treatments within each time period of 7 or 14 days followed by different letters in upper case are significantly different ($P<0.05$, Tukey's test).

Table 2.4. Mean mite repellency indices for the liquid smoke preparations at 100%, 50%, 25%, 10%, 5%, 1% and 0.3%, coated on semi-moist pet food at enumeration time points 1 h, 8 h, and 24 h. Semi-moist pet food coated with distilled water served as the untreated control and 20% propylene glycol treatment served as the positive control. Positive control showed 100% repellency. RI values which are "+" indicate repellency and "–" indicate attraction.

Treatment	Time (h)	Repellency Index (Mean ± SE)[abc]						
		Concentration						
		0.3%	1%	5%	10%	25%	50%	100%
P-1720	2	-25 ± 5	10 ± 4	-20 ± 3	20 ± 6	-20 ± 3	-50 ± 4	-40 ± 5
	8	-30 ± 3	20 ± 6	-20 ± 3	0 ± 4	-20 ± 5	-40 ± 4	-40 ± 3
	24	-40 ± 3 a, B	0 ± 4 a, B	-30 ± 5 a, B	-10 ± 3 a, B	-30 ± 6 a, B	-40 ± 3 a, B	-40 ± 4 a, B
Cloud S-5	2	10 ± 6	0 ± 3	-10 ± 4	-20 ± 5	0 ± 4	20 ± 6	10 ± 3
	8	10 ± 4	10 ± 5	0 ± 4	-40 ± 3	-20 ± 6	20 ± 4	0 ± 5
	24	20 ± 3 a, AB	20 ± 6 a, AB	0 ± 3 a, AB	-50 ± 3 a, AB	-20 ± 4 a, AB	10 ± 3 a, AB	0 ± 6 a, AB
Cloud S-C100	2	-10 ± 5	10 ± 4	20 ± 6	30 ± 6	10 ± 3	50 ± 4	50 ± 5
	8	0 ± 3	10 ± 6	30 ± 4	30 ± 4	-10 ± 5	60 ± 4	50 ± 3
	24	10 ± 3 a, A	10 ± 4 a, A	25 ± 3 a, A	20 ± 3 a, A	-10 ± 6 a, A	60 ± 3 a, A	40 ± 3 a, A
Black deli	2	10 ± 3	0 ± 6	-10 ± 3	-20 ± 3	10 ± 4	-20 ± 3	0 ± 6
	8	-10 ± 4	10 ± 5	40 ± 4	-20 ± 3	-20 ± 6	0 ± 4	10 ± 5
	24	-20 ± 6 a, B	-50 ± 3 a, B	10 ± 4 a, B	-50 ± 5 a, B	0 ± 4 a, B	-20 ± 6 a, B	0 ± 3 a, B
Hickory OS-1473	2	-20 ± 5	20 ± 4	-50 ± 3	-20 ± 6	-40 ± 3	-20 ± 4	10 ± 5
	8	0 ± 3	-40 ± 4	-20 ± 5	-30 ± 3	-40 ± 6	20 ± 3	-20 ± 4
	24	0 ± 3 a, B	-10 ± 6 a, B	-40 ± 3 a, B	-30 ± 4 a, B	-40 ± 5 a, B	-30 ± 4 a, B	-40 ± 3 a, B
Code-10	2	-25 ± 5	10 ± 4	-20 ± 3	20 ± 6	-20 ± 3	-50 ± 4	-40 ± 5
	8	-30 ± 3	20 ± 6	-20 ± 3	0 ± 4	-20 ± 5	-40 ± 4	-40 ± 3
	24	-40 ± 3 a, B	0 ± 4 a, B	-30 ± 5 a, B	-10 ± 3 a, B	-30 ± 6 a, B	-40 ± 3 a, B	-40 ± 4 a, B
Code-V	2	10 ± 6	0 ± 3	-10 ± 4	-20 ± 5	0 ± 4	20 ± 6	10 ± 3
	8	10 ± 4	10 ± 5	0 ± 4	-40 ± 3	-20 ± 6	20 ± 4	0 ± 5
	24	20 ± 3 a, AB	20 ± 6 a, AB	0 ± 3 a, AB	-50 ± 3 a, AB	-20 ± 4 a, AB	10 ± 3 a, AB	0 ± 6 a, AB
Cloud S-AC15	2	-10 ± 5	10 ± 4	20 ± 6	30 ± 6	10 ± 3	50 ± 4	50 ± 5
	8	0 ± 3	10 ± 6	30 ± 4	30 ± 4	-10 ± 5	60 ± 4	50 ± 3
	24	10 ± 3 a, A	10 ± 4 a, A	25 ± 3 a, A	20 ± 3 a, A	-10 ± 6 a, A	60 ± 3 a, A	40 ± 3 a, A

[a]Each mean is based on $n = 3$ replications.

[b]Means within treatments across different concentrations at 24 h followed by different lowercase letters are significantly different ($P<0.05$, Tukey's test).
[c]Means among treatments at 24 h at each concentration followed by different uppercase letters are significantly different ($P<0.05$, Tukey's test).

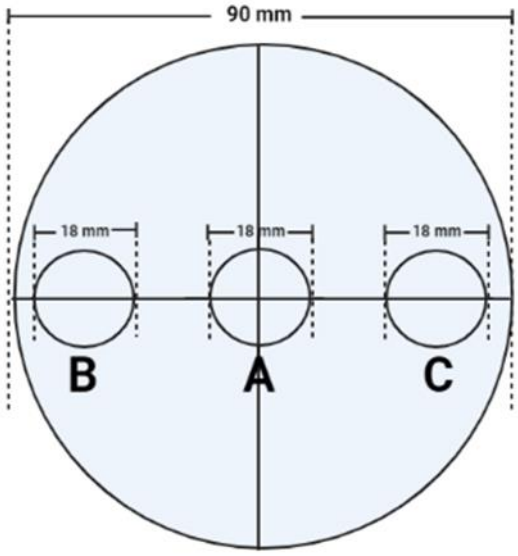


Fig 2.1. Bioassay arena used in two-choice behavioral test for mite orientation study. One 'treated' pet food cube and one 'control' pet food cube was placed in each of the circles, B and C. Twenty adult mites of mixed sex were released into the middle circle A.

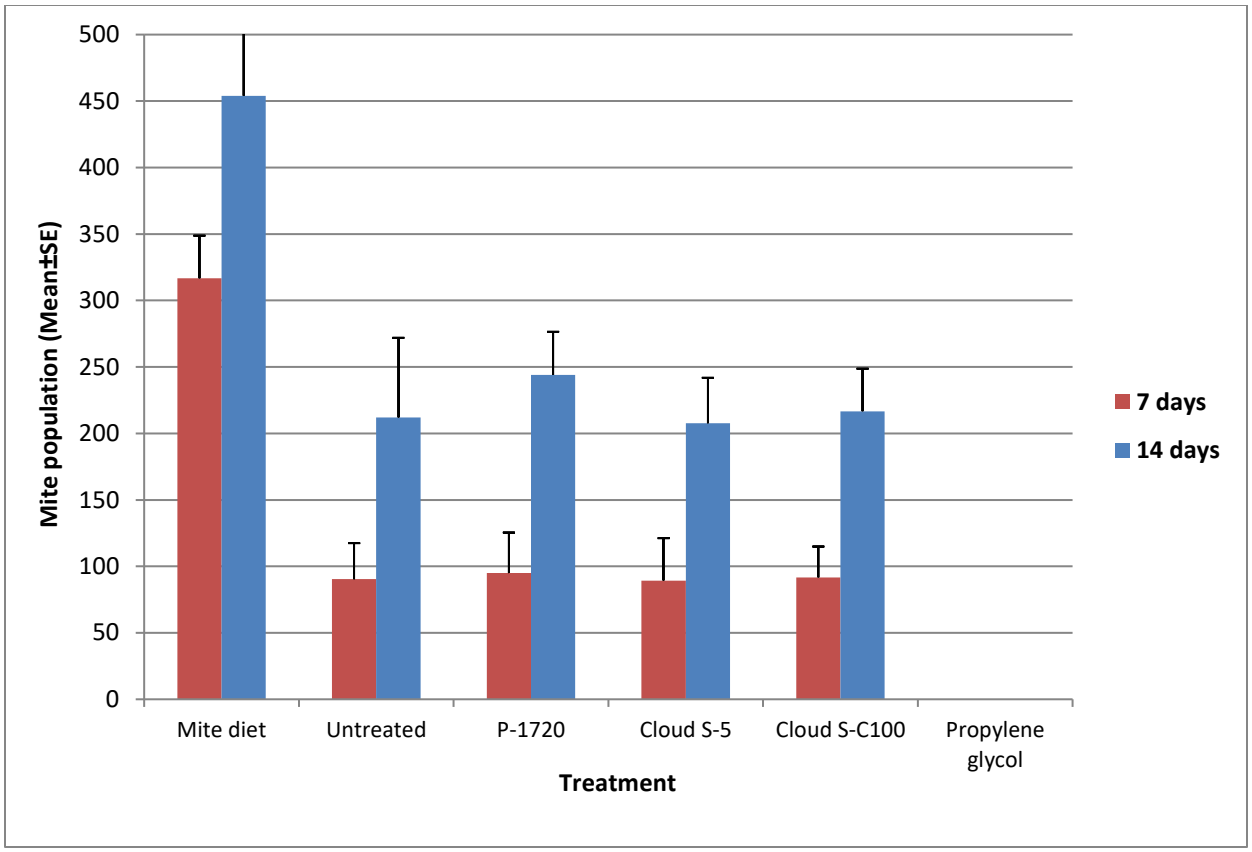
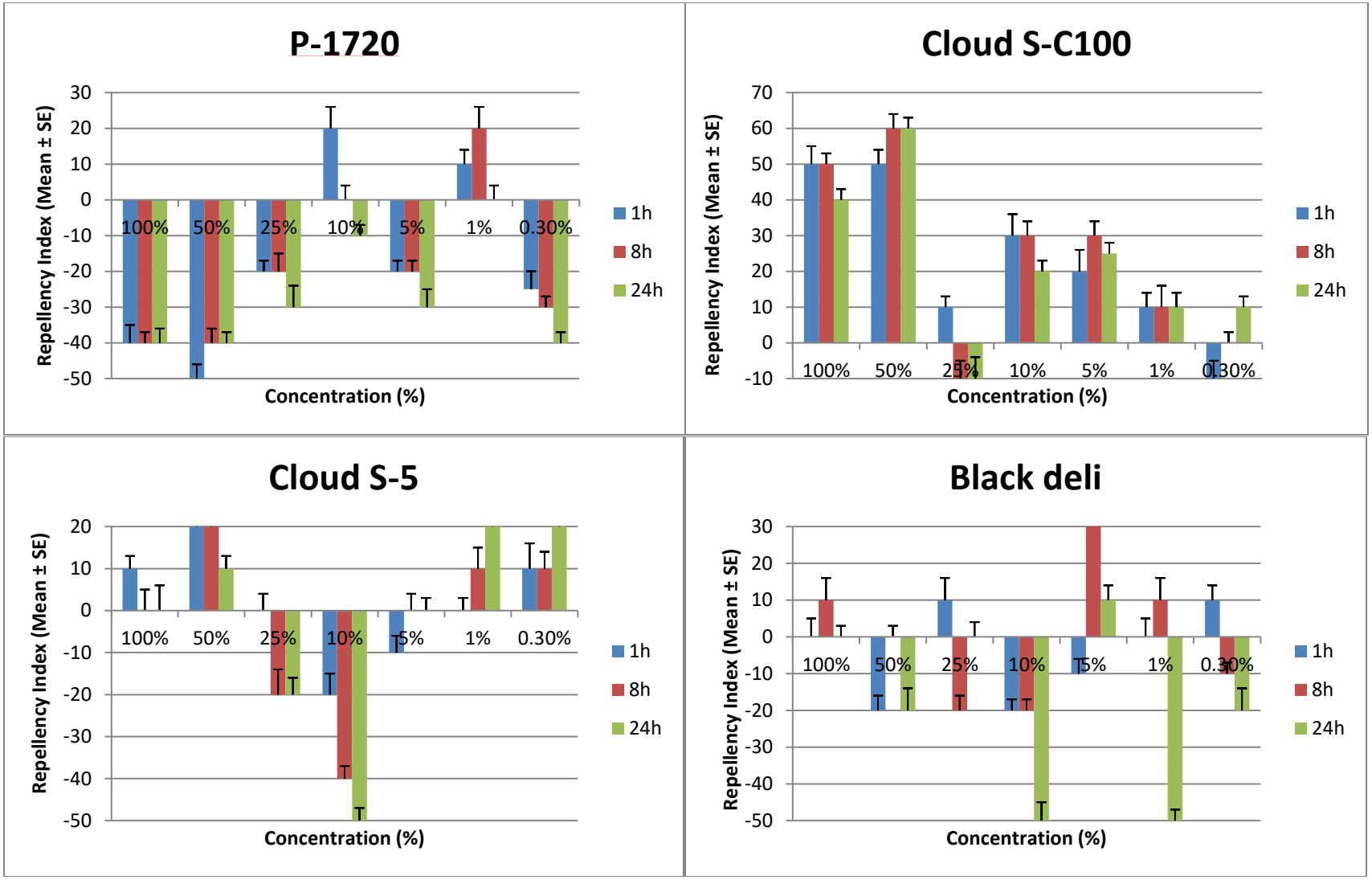


Fig. 2.2. Mean mite population growth at 7 and 14 days on semi-moist pet food treatments with inclusion of liquid smoke preparations namely P-1720, Cloud S-5 and Cloud S-C100 at 0.3% w/w, in comparison to the untreated control (semi-moist pet food with no smoke added), the standard mite rearing diet (lab culture diet), and 20% propylene glycol treatment (positive control).


P-1720
Repellency Index (Mean ± SE)
30
20
10
0
-10
-20
-30
-40
-50
100%
50%
25%
10%
5%
1%
0.30%
Concentration (%)
1h
8h
24h
Cloud S-C100
Repellency Index (Mean ± SE)
70
60
50
40
30
20
10
0
-10
100%
50%
25%
10%
5%
1%
0.30%
Concentration (%)
1h
8h
24h
Cloud S-5
Repellency Index (Mean ± SE)
20
10
0
-10
-20
-30
-40
-50
100%
50%
25%
10%
5%
1%
0.30%
Concentration (%)
1h
8h
24h
Black deli
Repellency Index (Mean ± SE)
30
20
10
0
-10
-20
-30
-40
-50
100%
50%
25%
10%
5%
1%
0.30%
Concentration (%)
1h
8h
24h

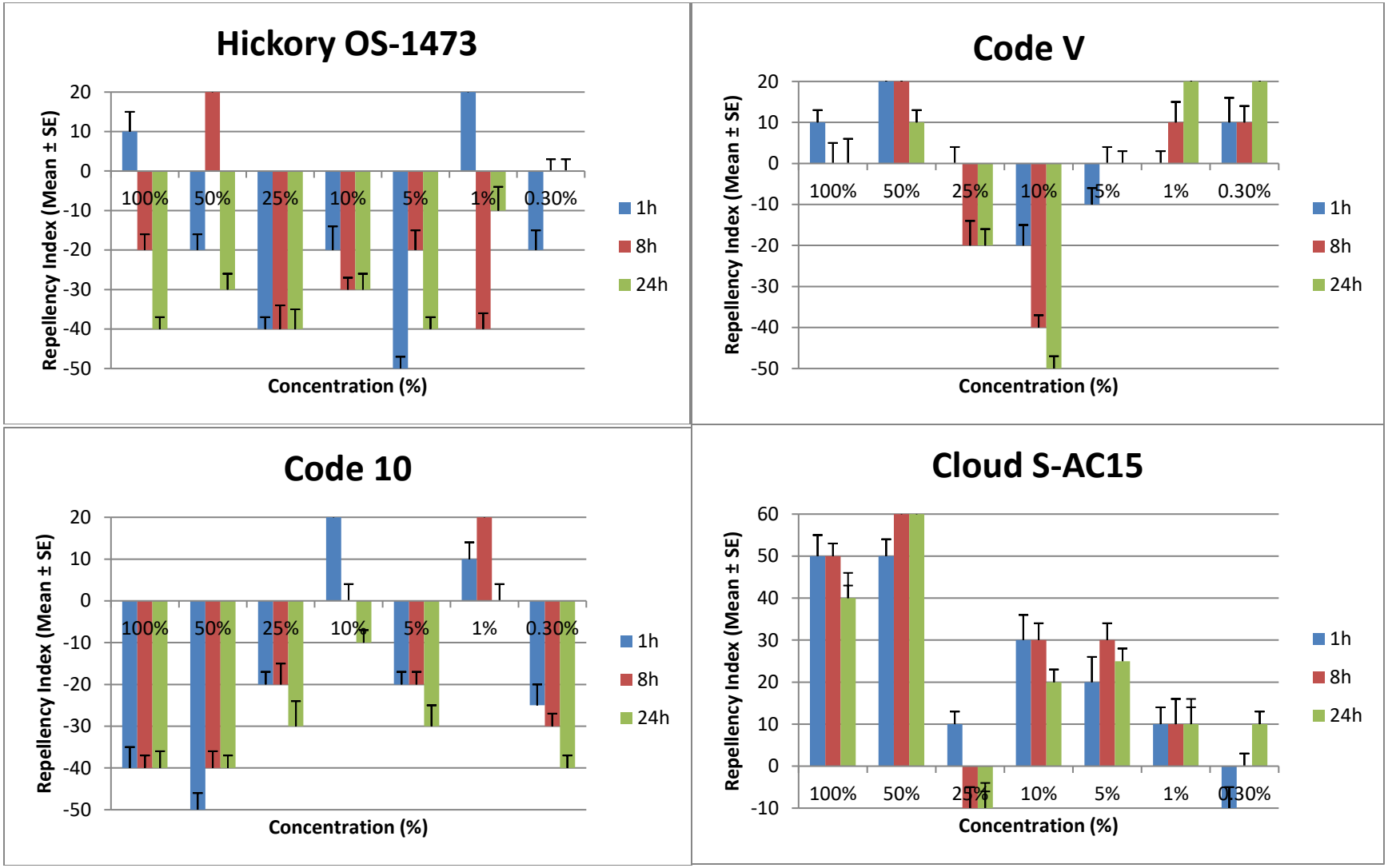


Fig. 2.3. Mean mite repellency indices (values which are "+" indicate repellency and "-" indicate attraction) for the liquid smoke preparations namely P-1720, Cloud S-5, Cloud S-C100, Black deli, Hickory OS-1473, Code 10, Code V and Cloud S-AC15 at concentrations 100%, 50%, 25%, 10%, 5%, 1% and 0.3%, coated on semi-moist pet food at enumeration time points 1 h, 8 h, and 24 h. Semi-moist pet food coated with distilled water served as the untreated control and 20% propylene glycol treatment served as the positive control.

Chapter 3 - Effects of liquid smoke preparations on shelf life and growth of wild-type mold and *Aspergillus flavus* in a model semi-moist pet food system

Abstract

Liquid smoke is a naturally derived flavor component and preservative with known antimicrobial properties. To our knowledge, there is a paucity of information on antimycotic potential of liquid smoke against storage fungi like *Aspergillus flavus* that produce mycotoxins in human and pet foods. Semi-moist pet food with high moisture content (20-30%) is susceptible to mold contamination and requires intervention. The objectives of this study were to determine the effects of liquid smoke preparations on the growth of wild-type mold and *A. flavus* in semi-moist pet food. Semi-moist pet food was formulated with liquid smoke preparations (S1 to S8) at 0% (untreated), 0.5%, 1%, 2% and 4% (w/w). A positive control consisted of 1% potassium sorbate known to inhibit mold growth and no smoke. Shelf life was estimated by storing the samples at 28°C and 65-70% RH over 30 days and recording the number of days until appearance of visible wild-type mold. In another experiment, samples were spot inoculated with *A. flavus* (~10,000 CFU/mL), incubated at 25°C, and analyzed for fungal growth by enumeration on potato dextrose agar, at sampling intervals of 2 days over a 35-day period. Liquid smoke at 0.5%, 1%, 2%, and 4% extended the shelf life of samples by 11.6, 12.5, 17.2, and 24.1 days when compared to the untreated samples (7.7 days). The smoke preparations Cloud S-C100 and Code-10 (high carbonyl, medium/low phenol) were the most effective in prolonging the number of days to visible mold growth (26-28 days). In the challenge study with *A. flavus*, Cloud S-C100, Cloud S-AC15 (high to medium carbonyl, low phenol), and Code 10 (base smoke) reduced mold counts by 1.0, 1.7, and 2.5 logs when compared to the untreated samples at 1, 2, and 4% respectively. Addition of smoke at 0.5% did not reduce mold counts. The carbonyl preparations of liquid smoke were the most effective at enhancing shelf life of semi-moist pet food, and at inhibiting *A. flavus* growth.

Key words: Liquid smoke, Semi-moist pet food, *Aspergillus flavus*, Mold challenge

Introduction

One of the risk factors for human and animal food safety is the presence of fungi and the potential for mycotoxins that they may produce. Hazardous mycotoxins can occur in cereal grains due to stress during growth, can be compounded with improper storage, and the process of cooking does not reduce their content (Cinar et al., 2019). During manufacturing, food can be contaminated with mold spores especially when cereal grains are ground, and the foods are pelleted or formed (Almeida et al., 2011). Mold spores prevalent in the environment also contaminate packaged foods that are opened by the consumer and may amplify during food storage. Mold growth in foods ultimately causes spoilage leading to food waste. Moldy foods also reduce the nutritional value (Bluma et al., 2008) and under certain conditions may produce toxic metabolites called mycotoxins. These toxins with chemically diverse structures have been involved in disease outbreaks which have affected both animal and human health (Beardall et al., 1994; Prelusky et al., 1994).

Semi-moist pet food products contain meat, starch-based ingredients such as grains, and fats/oils. They are sold at a moisture content of approximately 20-30%. This makes them conducive to mold growth during storage if water activity is left uncontrolled. Thus, an intervention strategy is needed to retard food spoilage and potential food safety concerns. The primary method to control water activity is to use humectants, polyols or sugar alcohols like propylene glycol and glycerin. Glycerin is considered a natural ingredient while propylene glycol is synthetic. Propylene glycol apart from controlling water activity is also effective at eliminating infestation of mites in semi-moist foods, whereas glycerin is the preferred natural humectant, but it can attract mites. Therefore, to augment control of mold growth even when water activity is controlled, a mold inhibitor such as potassium sorbate is typically included. Potassium sorbate is

synthetic, and a natural alternative is desired for 'clean labeling' of pet foods in accordance with the current consumer preference trend.

Liquid smoke is a naturally derived flavor component and preservative used in human and pet foods, with known antimicrobial properties (Maga 1988; Sunen et al., 2003; Lingbeck et al., 2014;). The commercial production of liquid smoke involves the pyrolysis process whereby thermal decomposition of wood at 10% moisture and at 400-500°C in retorts or rotary ovens under absence of oxygen captures the resulting smoke after a water spray that separates the light and heavy fractions by gravity (Borys, 2004). The liquid smoke flavorings are usually obtained by fractionating the resulting condensate through sequential extraction or liquid-liquid partitioning and/or solid-phase extraction technique based on the polarity and acidity of the constituents. Liquid smokes in the food processing industry are used as flavoring agents, browning colorants, antioxidants, food texture enhancers and as antimicrobial agents (Maga, 1988; Borys, 2004; Coronado et al., 2012; Sunen et al., 2012).

Liquid smoke and its fractions containing phenols, carbonyls and organic compounds have been found to be effective against pathogenic bacteria like *Listeria monocytogenes* and *Staphylococcus aureus* (Sunen et al., 2003; Lingbeck et al., 2014) in meat and fish products. Studies evaluating their effects against fungi or molds in food substrates have been limited. The use of liquid smoke to impregnate fibrous cellulosic food/meat casings to prevent mold growth was found to be effective by Chiu (1983) (U.S. Patent 4377187). Wendorff et al. (1993) evaluated the effects of liquid smoke on the growth of *Aspergillus oryzae, Penicillium camemberti*, and *Penicillium roqueforti* on cheddar cheese. They found that liquid smoke applied to the surface of cheese inhibited growth of *A. oryzae and* increased the lag period of *P. camemberti* and *P. roqueforti.* Milly et al. (2005) evaluated the phenol and carbonyl fractions of liquid smoke in

nutrient broth against the mold *A. niger* and reported their minimum inhibitory concentrations to be in the range of 1.5% to 5%. Beyond these we could not find any available scientific literature on the antimycotic potential of liquid smoke in food matrices against common storage molds such as *Aspergillus* and *Fusarium* spp. which produce mycotoxins (aflatoxin and deoxynivalenol, respectively) in food and feed. Further, mold spoilage is visible and undesirable by consumers resulting in food waste. The objectives of this research study were: (i) to investigate the effects of liquid smoke preparations on storage fungi, *Aspergillus flavus* and *Fusarium graminearum,* by determining their minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) in nutrient broth; (ii) to determine the number of days to observe wild-type mold growth in semi-moist pet food prepared with liquid smoke inclusions at various concentrations using a shelf-life model study; and (iii) to evaluate the effects of liquid smoke preparations on the growth of *A. flavus* in semi-moist pet food using a mold challenge study.

Materials and Methods

Fungal cultures

Aspergillus flavus (ATCC 15548) and *Fusarium graminearum* (ATCC 56091) procured from the American Type Culture Collection (ATCC, Manassas, VA, USA) were maintained in potato dextrose broth (PDB)-glycerol (7:3) at -80°C. Before use, the frozen stock cultures were thawed and streaked on potato dextrose agar (PDA; Difco Laboratories, Sparks, MD, USA) plates and incubated at 28°C for 72 h. Chloramphenicol (75 mg/L) was added to PDA to prevent the growth of bacteria. The fungal spores were collected from the grown culture on PDA by adding 5 mL of potato dextrose broth (PDB; Difco Laboratories, Sparks, MD, USA) to the surface of the dish. The spores were then dislodged from the solid medium using an L-shaped plastic rod. The

spore suspension in nutrient broth was then collected and stored at 4°C and used as the fungal inoculum when needed.

Preparation of semi-moist pet food and source of liquid smoke

The formula for the model semi-moist pet food was modified from the previous study mentioned in Chapter 2. The modified formula used for this study is shown in Table 3.1. All the ingredients were weighed according to the formula (Table 3.1), to produce 2 kg of pet food per batch. A 3.3 L planetary mixer (KitchenAid Portable Appliances, St. Joseph, MI, USA) was used to mix the ingredients at a speed of 50 rpm for 10 min. The dry ingredients were mixed first, followed by the liquid ingredients. The mixture was spread in a uniform layer of approximately 1 cm thickness on a baking tray lined with parchment paper. It was baked in a convection oven (MEA 21-93-E; Garland Commercial Industries, PA, USA) at 175°C for 10 min. The baked pet food was then cooled on a wire rack to room temperature. It was then cut into uniform cubes of size 3×3×1 cm using a stainless-steel knife.

Eight liquid smoke preparations (Table 3.2; Kerry Inc., Beloit, WI, USA), S1, S2, S3, S4, S5, S6, S7, or S8 were evaluated at 0.5, 1, 2 and 4% w/w inclusion in semi-moist pet food for experiments 2 and 3 of this study. The initial moisture content of the pet food samples was 25% dry basis. Water activity (a_w) and pH were recorded and reported in Table 3.3. Semi-moist pet food made with 1% potassium sorbate and containing no smoke served as the positive control, which is known to inhibit mold growth, and with no potassium sorbate and no smoke served as the 'untreated' control.

Experiment 1: Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

The minimum inhibitory concentrations (MIC) of the liquid smoke treatments were determined by the broth micro- and macro-dilution assay according to the antimicrobial susceptibility testing methods described by the Clinical and Laboratory Standards Institute (CLSI, 2014). To determine MIC, a 200 µL volume of liquid smoke preparation (S1, S2, S3, S4, S5, S6, S7, or S8) consisting of twice the desired final concentration were dispensed in the first well of a 96-well microtiter plate and 100 µL of sterile water in rest of the wells. A serial two-fold dilution of the liquid smoke preparations was performed starting from 50% to 0.05% v/v concentration. A 100 µL aliquot of fungal (*Aspergillus flavus* or *Fusarium graminearum*) culture (~10,000 CFU/mL) was added to each well of the plate already containing the 100 µL of decreasing concentrations of liquid smoke preparations, to make a final volume of 200 µL per well. The positive control consisted of fungal inoculum only (no treatment), and the negative control consisted of potato dextrose broth (PDB) alone (no treatment, no inoculum). The MIC was determined as the lowest concentration of liquid smoke that inhibited visible growth of *A. flavus* or *F. graminearum* in the microtiter plate after 72 h of incubation at 28ºC. Inhibition of mold growth was visually assessed by absence of mycelial growth or turbidity in the plate wells over a period of 30 days of incubation at 28ºC (Lopez-Malo et al., 2007). A similar experiment was also conducted with potassium sorbate as treatment in the place of liquid smoke to determine its MIC against *A. flavus* and *F. graminearum*. The study was replicated three times.

To determine minimum fungicidal concentration (MFC), 100 µL of sample from each well from the MIC experiment was plated on potato dextrose agar (PDA) for enumeration of fungal colonies at each concentration of treatment (liquid smoke or potassium sorbate) after incubation

at 28ºC for 72 h. MFC was determined as the lowest concentration of treatment that caused absence (≤ 1 colony) of fungal growth on the agar plate.

Experiment 2: Days-to-mold (shelf life) study

For this study, the liquid smoke preparations S1, S2, S3, S4, S5, S6, S7, or S8 were evaluated at 0.5, 1, 2, and 4% w/w inclusion in semi-moist pet food. Semi-moist pet food with no potassium sorbate and no smoke served as the untreated control. Semi-moist pet food made with 1% potassium sorbate and containing no smoke served as the positive control, known to inhibit mold growth. About 30 g of sample from each treatment was placed into a Whirl-Pak bag (Nasco, Ft. Atkinson, WI, USA) each with four pin holes, and placed in an environmental chamber at 28°C and 65-70% relative humidity in total darkness. The samples were observed every day over a period of 30 days for visible mold growth on the surface. The number of days for the first mold colony to appear on the surface of the pet food cubes for each sample was recorded. The study was replicated three times.

Experiment 3: Mold challenge study with *Aspergillus flavus*

The fungal inoculum from cultures of *Aspergillus flavus* (ATCC 15548) were prepared as described earlier. A volume of 1 mL of the inoculum (~10,000 CFU/mL) was spot applied on 25 g of semi-moist pet food treatment (described earlier) using a pipette. The initial moisture content of the pet food samples was 25% and the final moisture content post-inoculation was maintained at ~29%. The inoculated samples were stored in Whirl-Pak bags at 28°C. They were analyzed for fungal growth at a sampling interval of 2 days over a period of 35 days (day 0, day 1, day 3, day 5 etc. up to day 35). For the fungal analysis, the treatment samples (25 g) were placed in sterile Whirl-Pak bags and mixed in 225 mL of buffered peptone water (Difco Laboratories, Sparks, MD, USA) and stomached for 2 min. The mixtures were serially diluted in 0.1% peptone water (Difco

Laboratories, Sparks, MD, USA) and plated on potato dextrose agar (PDA). Chloramphenicol (75 mg/L) was added to PDA to prevent the growth of bacteria. The plates were incubated at 28°C for 72 h and then the fungal colonies were counted. The fungal counts were expressed as log CFU/mL.

Statistical analysis

For the days-to-mold study, the mean number of days taken to grow mold for the treatments (untreated (0%), smoke treatments S1 to S8) at concentrations 0.5%, 1%, 2% and 4% were subjected to two-way analysis of variance (ANOVA) using the GLIMMIX procedure of SAS version 9.3 statistical software, with means among treatments separated using Tukey's test ($P \leq 0.05$) (SAS Institute, 2011). For the mold challenge study with *A. flavus*, the mean log reductions of mold counts between day 0 and day 35 for the treatments (untreated (0%), smoke treatments S1 to S8) at concentrations 0.5%, 1%, 2% and 4% were also subjected to two-way ANOVA, with means among treatments separated using Tukey's test ($P \leq 0.05$) (SAS Institute, 2011). The linear model $y = a + bx$ was fit to logarithmic reduction of mold counts over time (days) for the smoke treatments S1 to S8 at 2% and 4%, where a is the intercept and b is the slope. The decimal reduction time or D-value (time taken for 1-log reduction of mold counts) was calculated as the negative-inverse of slope (Mazzola et al., 2003).

Results

The MICs and MFCs of liquid smoke preparations and potassium sorbate against *Aspergillus flavus* and *Fusarium graminearum* are shown in bar graphs (Figures 3.1 and 3.2). The MICS of liquid smoke ranged from 1.6 to 6.3% for *A. flavus,* and 0.4 to 1.6% for *F. graminearum*. The MFCs of liquid smoke ranged from 3.1 to 12.5%, and 0.4 to 3.1%, for *A. flavus* and *F. graminearum,* respectively. For potassium sorbate, the MIC as well as the MFC were 0.195% and 0.098% for *A. flavus* and *F. graminearum,* respectively. Against *A. flavus,* the liquid smoke

preparations Cloud S-C100, Code-10, and Cloud S-AC15 had the lowest MIC (1.56%), and P-1720, Cloud S-C100, and Cloud S-AC15 had the lowest MFC (3.125%). Against *F. graminearum*, P-1720, Cloud S-C100, Code-10, and Cloud S-AC15 had the lowest MIC (0.39%), and Cloud S-C100 and Cloud S-AC15 had the lowest MFC (0.39%). The least effective liquid smoke preparations against *A. flavus* were Cloud S-5 and Hickory OS-1473 with the highest MIC (6.25%) and MFC (12.5%). Against *F. graminearum*, the least effective liquid smoke preparations were Cloud S-5 and Black deli with the highest MIC (1.56%), and Cloud S-5 with the highest MFC (3.125%).

The mean number of days to mold for the untreated and smoke treated samples are reported in Table 3.4 and Fig. 3.3. The positive control (1% potassium sorbate, no smoke) did not allow mold growth during the observation period of 30 days. The untreated (no smoke treatment) sample allowed mold growth in 7.7 days. The average days to grow mold for the smoke treatments at 0.5% ranged from 8.7 (Code V) to 13.3 days (Cloud S-C100 and Code 10), at 1% ranged from 9.3 (Code V) to 15.3 days (Black deli), at 2% ranged from 14.3 (Code 10 and Cloud S-5) to 21.0 days (Hickory OS-1732 and Cloud S-AC15), and at 4% ranged from 18.0 (Black deli) to 28.0 days (Cloud S-C100 and Code-10). Grouping of treatment means (Table 3.4) by Tukey's post-hoc test (F = 41.04; df = 11, 87; P<0.05) indicated that among the smoke treatments, Cloud S-C100 and Cloud S-AC15 were the most effective in prolonging the number of days to mold detection whereas Code-V and Cloud S-5 were the least effective. Within each smoke treatment, except for Black deli, there was no difference between 0.5% and 1% concentrations, and 4% concentration was the most effective in prolonging the shelf life of pet food samples.

Reduction in mold counts of *Aspergillus flavus* was observed due to liquid smoke inclusion in the semi-moist pet food samples as shown in Table 3.5 and Fig 3.4. The untreated sample (0%

smoke) had an increase in mold counts up to 5 logs from an initial load of 3.5 logs over the 35 days incubation period. Across all treatments, inclusion of liquid smoke at 4% decreased (P<0.05) mold counts over time compared to 0, 0.5, 1, and 2%. The mean mold count log reduction for the smoke treatments, between day 0 and day 35 at 4% ranged from 1.5 logs (Code V) to 2.5 logs (Cloud S-C100), at 2% ranged from 0.2 logs (Black deli) to 1.9 logs (Cloud S-C100), and at 1% ranged from 0.6 logs (Cloud S-C100) to 1.3 logs (Cloud S-AC15). At 0.5% none of the smoke treatments had any log reduction in mold counts and instead showed an increase of 0.1 logs (Black deli) to 0.7 logs (Cloud S-C100). Also at 1%, the smoke treatments P-1720, Cloud S-5, Black deli, Hickory OS-1473, and Code-V experienced a logarithmic increase in mold counts (0.1 - 0.8 logs), and at 2% there was an increase of 0.1 - 0.6 logs for P-1720, Cloud S-5, Hickory OS-1473, and Code-V. Across the different time points, mold count reductions were observed after 16 days (P<0.05) on average across the smoke treatments. Within each smoke treatment, there was no difference between 0.5% and 1% concentrations for P-1720, Black deli, and Hickory OS-1473, no difference between 1% and 2% concentrations for P-1720, Cloud S-C100, Hickory OS-1473, Code-V, and Cloud S-AC15, and 4% concentration was the most effective (P<0.05) at reducing mold counts. Among the smoke treatments, Cloud S-AC15, Cloud S-C100, and Code-10 were the most effective (P<0.05) in reducing mold counts, whereas P-1720 and Cloud S-5 were the least effective.

The *D*-values, which is the time in days required to record 1-log reduction of mold counts, for each treatment are shown in Table 3.6. At 4% concentration of smoke, the *D*-values ranged from 5 days (Cloud S-C100) to 14.3 days (Code-V), and at 2% the *D*-values ranged from 16.7 days (Code-10) to 50 days (Cloud S-5). *D*-values were not calculated for treatments showing no log

reduction of mold counts over time. Among the smoke treatments, Cloud S-C100 and Cloud S-AC15 were the most effective in reducing *A. flavus* counts with the lowest *D*-values (5-9 days).

The linear regression model $y = a + bx$ was best fit for the 4% treatments with higher R^2 values (>0.70) due to more pronounced log reductions of the mold counts. The model fit was poor (low R^2 values) for most of the 2% smoke treatments and so *D*-values could not be obtained for several of the smoke treatments at 2% due to no consistent log reductions of mold count over time. However, it shows a fungistatic effect of the liquid smoke inhibiting the mold growth.

Discussion

In vitro microbial susceptibility tests such as MIC and MFC, are usually performed to evaluate the sensitivity of an organism to an antimicrobial agent such as an antibiotic or chemical preservative. From the MIC and MFC assays in this study, Cloud S-C100 and Cloud S-AC15 were found to be the most effective against both the fungal species in nutrient broth. The least effective were Cloud S-5, Black deli and Hickory OS-1473. This study points out that liquid smoke preparations containing medium to high carbonyl content (Cloud S-C100 and Cloud S-AC15) were more effective at inhibiting fungal growth (fungistatic or fungicidal) in broth dilution antimycotic susceptibility tests, while those containing low acid & buffered pH or high phenol content (Cloud S-5, Black deli and Hickory OS 1473) were the least effective. MIC and MFC of liquid smoke depend on several variables including composition and concentration of its components such as phenols, carbonyls and organic acids and the cultural conditions for the test organisms, and so comparison of results with those of other studies involving smoke is not simple. Studies concerning smoke condensates and their antimicrobial potential have been performed before (Maga 1988; Sunen 1988; Estrada-Munoz et al., 1998; Painter 1998; Sunen et al., 2001; Sunen et al., 2003). These previous studies were done using smoke preparations having a different composition than

the ones used in the present study and concluded that phenols are responsible for the antimicrobial properties of smoke condensates. In this study we tested smoke preparations with a range of phenol, carbonyl, and organic acid contents to determine the effectiveness of the combination of these components as antimicrobial agents. Our MIC results (1.56% - 6.25%) are in accordance with the results reported by Milly et al. (2005) who determined the minimum inhibitory concentrations of phenol and carbonyl fractions of liquid smoke in nutrient broth against yeast (*Saccharomyces cerevisiae*) and mold (*A. niger*) and reported the MIC of *A. niger* to be in the range of 1.5 to 5%. The MIC and MFC assays in this study indicate that across all treatments, the mold *F. graminearum* was more susceptible to antimycotic or antifungal effects of liquid smoke than *A. flavus*. From the studies by Panwar et al. (2016) and Casquete et al. (2017) it was reported that *Aspergillus flavus* is more tolerant of fluctuations in growth conditions like temperature and pH and is a more 'robust' mold than *F. graminearum*. This may explain why *Fusarium* was more prone to antimycotic effect from the liquid smoke than *Aspergillus*. This was also the reason the mold challenge experiments in this study using liquid smoke preparations in semi-moist pet food was conducted using only *A. flavus*. While comparing the MIC and MFC concentration results in this study, it can be found that the MFC is almost twice that of the MIC, and in some cases the same as MIC, across the different treatments. According to Pankey et al. (2004) when the ratio of minimum bactericidal concentration (MBC) to MIC is less than or equal to 4, the antimicrobial can be considered bactericidal. Since the MFC to MIC ratios of liquid smoke for *A. flavus* and *F. graminearum* in this study is in the range of 1 to 2, it can be considered as fungicidal and should be effective against these molds when applied in food systems (Kiprotich et al., 2021). Based on the MIC and MFC results of liquid smoke preparations from experiment 1 of this study, semi-

moist pet food was prepared with smoke inclusions at 0.5, 1, 2, and 4% (w/w) and a shelf-life model study was conducted as experiment 2 to determine the number of days to mold.

In the shelf-life model study, when compared to the untreated semi-moist pet food, the smoke treatments prolonged the number of days to significant mold. Overall, at 4% concentration the liquid smoke preparations extended the shelf life of semi-moist pet food samples by 20 days or more, with the highest being 26-28 days as seen in Cloud S-C100 and Cloud S-AC15, however at 0.5% the shelf life was prolonged by only ~5 days, with the highest being 12-13 days (Cloud S-C100 and Cloud S-AC15) in comparison with untreated sample's shelf life (7.7 days). Among the smoke treatments averaging across concentrations, Cloud S-C100 and Cloud S-AC15 containing medium to high carbonyl content were the most effective in prolonging the number of days to mold whereas Code-V (organic acid preparation of liquid smoke) and Cloud S-5 (buffered pH, low acid preparation) were the least effective. These results show that high carbonyl content in liquid smoke has a fungicidal effect on wild-type environmental mold, unlike that reported by some previous studies (Maga 1988; Sunen 1988; Estrada-Munoz et al., 1998; Painter 1998; Sunen et al., 2001; Sunen et al., 2003) which attributed the antimicrobial effect of smoke to be the phenol content. Deans et al. (1987), and Dorman et al. (2000) who studied antimicrobial activity of plant essential oils and Tayel (2010) who studied antifungal effects of smoke from smoldering medicinal plants like cinnamon on *A. flavus* also attributed the antimicrobial and antifungal properties to compounds containing phenolic groups present in the plants and their essential oils. Our results showed that carbonyl components in liquid smoke have antimycotic activity against environmental molds. The proposed mechanism of action of carbonyl compounds in liquid smoke against micro-organisms or bacterial cells was explained by Lingbeck et al. (2014). Carbonyls inhibit microbial growth by penetrating the cell wall and inactivating enzymes located in the cytoplasm and the

cytoplasmic membrane (Milly, 2003). Carbonyls act by condensing with the free, primary amino-groups in the polypeptide chains, primarily in the side chains of basic amino-acids. These amino groups may be an essential part of active site of the enzyme, or they may function to bind the substrate by hydrogen-bonding (Painter, 1998). Even if the carbonyls cannot access the interior of a microbial cell, they can still inhibit growth by interfering with the uptake of nutrients.

The water activity of semi-moist pet foods usually ranges from 0.6-0.8 a_w and pH is about 5. The pH of each of the liquid smoke preparations used in this study varied considerably (ranging from acidic to alkaline) but pH of the semi-moist pet food treatments with inclusion of liquid smoke in this study ranged from 4.6-5.3 (Table 3.3). This higher range of pH is possibly due to buffering effect of mineral salts present in the pet food formula. So, we believe the low pH effect is not contributing substantially to the antifungal properties of liquid smoke; instead, it could be the synergistic action of carbonyls/organic acids/phenols present. More studies are required to investigate the action of carbonyl compounds in liquid smoke against molds and bacteria and any synergistic effect with organic acids and phenolic compounds.

To better understand the fungicidal or fungistatic potential of liquid smoke preparations in a model food substrate, i.e., semi-moist pet food over time, a mold challenge study with *A.flavus* was conducted as experiment 3 in this study with liquid smoke inclusions at 0.5, 1, 2, and 4% w/w and enumerating the mold counts over a 35-day period. When comparing the initial inoculated mold counts (day 0) with the final mold counts on day 35, the log reductions for Cloud S-C100, Cloud S-AC15, and Code 10 at 4% were 2.3-2.5 logs, at 2% were 0.9-1.9 logs, at 1% were 0.6-1.3 logs, and at 0.5% no log reductions were observed (Table 3.5). Among the smoke treatments, Cloud S-AC15, Cloud S-C100, and Code-10 were the most effective in reducing mold counts, whereas P-1720 and Cloud S-5 were the least effective. These results indicate that high to medium

carbonyl content (Cloud S-C100 and Cloud S-AC15) and base smoke (Code-10) preparations of liquid smoke had an antimycotic effect on *A. flavus* growth over time, whereas buffered pH smoke preparations like P-1720 and Cloud S-5 were the least effective against *A. flavus*. In our study, the mold counts over the 35-day incubation period of the various smoke treatments (Fig 3.5) indicate that even though there were no pronounced logarithmic reduction of mold counts for the lower concentrations 0.5 and 1% of smoke treatments, the *A. flavus* counts did not increase compared to the untreated samples. This shows a fungistatic effect of the smoke preparations. This result can be explained by the prolonged lag phase in the growth curves of *A. niger* when exposed to liquid smoke in the study by Milly et al. (2005). As they tested the effects of liquid smoke only in nutrient broth and not in any food substrate, our mold challenge study in model semi-moist pet food helps us in further understanding the antimycotic effects of applied liquid smoke. Milly et al. (2005) reported that high carbonyl and low pH fractions of liquid smoke were effective at inhibiting or prolonging lag phase in the growth curves of the tested micro-organisms including *A. niger*. Low pH effect of liquid smoke could not be correlated with the antimycotic effect in our study because even though the liquid smoke may have acidic pH, the pH in the semi-moist food substrate appeared to have been buffered due to the presence of mineral salts (as shown in Table 3.3).

The *D*-values (Table 3.6) for *A. flavus* exposed to liquid smoke at 2% and 4% concentrations in semi-moist pet food represent the times required for a 10-fold (1.0 log or 90%) destruction of the initial viable population of the mold. The decreases in *D*-values for the smoke treatments at 2 and 4% indicate a faster death rate of *A. flavus* at these concentrations in comparison to the untreated which did not have mold count reductions. The *D*-values also indicated faster death rate of *A. flavus* at 4% smoke concentration when compared to 2%. Even though *D*-values could not be obtained for several of the smoke treatments at 2% due to no consistent log reductions

of mold count over time, it showed fungistatic effect of the liquid smoke inhibiting the mold growth.

Most of the pathogenic bacteria stop growing at water activities less than 0.87 while the growth of common spoilage yeasts and molds stops at 0.70 a_w. Only xerophilic and osmophilic organisms can grow below 0.70 a_w and all microbial growth stops at water activities <0.60. For a food product to be considered shelf stable, its water activity must be less than 0.86 to ensure that no pathogenic bacteria will be able to grow on the product as it sits on the shelf. Foods with a water activity higher than 0.70 but less than 0.86 are considered unfavorable for bacterial growth but will still support the growth of mold and yeast which affects the shelf-life stability of the product (Scott, 1957; Beuchat, 1983). Accordingly, semi-moist pet food products with a typical water activity of 0.65-0.70 require a_w to be controlled using humectants like polyols and to prevent mold growth require the use of preservatives like potassium sorbate at 0.1%. In our study, the experimental semi-moist pet food that we manufactured and tested had a water activity of 0.76 which is higher than normal, therefore we expect to see a higher fungistatic or fungicidal effect when liquid smoke is applied to commercially manufactured semi-moist pet foods. Because consumers do not prefer having synthetic preservatives in their foods or pet foods for reasons described earlier, we suggest the use of liquid smoke as a natural preservative in semi-moist pet food.

The primary intention of smoking foods using a liquid form is to induce both a sought-after flavor and preservative effect. In addition, there may be microbiological effects of the applied smoke condensates. Liquid smoke is recognized as a GRAS (Generally Recognized as Safe) substance. This classification means that it can be used by food manufacturers without the need for a pre-market review verifying its safety. Furthermore, the use of GRAS substances is permitted as long as it is used in accordance with the manufacturers' Good Manufacturing Practices (GMPs)

(Rivera et al., 2021). Advantages and benefits associated with the use of liquid smoke in foods include ease and consistency of application to optimize antioxidant potential, sensory properties, and antimicrobial properties. Liquid smoke preparations can be easily controlled and evaluated for composition and consistency of application. Using condensates for smoke application allows the processor to dictate the concentration of smoke being applied more readily than using gaseous smoke (Sunen et al., 2001). The applied smoke can be evaluated for flavor acceptability in the product to determine the most suitable concentration. The antioxidant potential of smoke condensates has also been extensively documented, and its potential to retard lipid oxidation in many meat products is an added benefit (Maga 1988; Estrada-Munoz et al., 1998). Due to these reasons, liquid smoke can be readily used in semi-moist pet food as well as other intermediate moisture food applications.

Conclusion

It can be concluded from the results of our study that liquid smoke preparations which had high to medium carbonyl content namely Cloud S-C100 and Cloud S-AC15 at concentrations of 2 and 4% (w/w) inclusion in a model semi-moist pet food can have fungicidal or fungistatic effects against storage molds like *A. flavus*. It can potentially be used as a substitute for synthetic mold inhibitors like potassium sorbate to some extent. We believe that liquid smoke inclusion at 2% in pet food can be a more feasible option compared to the 4%, which might be too high of a level for addition as a flavor component. The liquid smoke concentration levels in pet food should be potentially evaluated in palatability tests for pets (dogs and cats) for practical applications.

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Tables & Figures

Table 3.1. Formula used for manufacturing the model semi-moist pet food.

Ingredient	Percent % w/w
Water	18.0
Corn	12.5
Chicken by-product meal	12.5
Corn gluten meal	12.5
Glycerin	12.5
Wheat	5.8
Choice white grease	5.8
Corn syrup	5.0
Gelatin	2.5
Rice flour	4.3
Soybean meal	4.3
Molasses	1.0
Dry dog digest (flavor)	0.5
Salt	0.5
Dicalcium phosphate	1.4
Vitamin premix	0.2
Potassium chloride	0.2
Lysine hydrochloride	0.1
Trace mineral premix	0.1
Calcium carbonate	0.1
Choline chloride	0.1
Natural antioxidant	0.1
Total	100.0

Table 3.2. Liquid smoke preparations evaluated in the study.

Smoke preparation	Name	Description
S1	P-1720	Buffered low phenol smoke, medium carbonyl
S2	Cloud S-5	Buffered pH, low acid, low carbonyl, no phenol
S3	Cloud S-C100	Carbonyl preparation: high carbonyl, low acid, very low phenol
S4	Black deli	Basic pH smoke, zero carbonyls, organic acid salts, phenols
S5	Hickory OS-1473	Phenol preparation: high phenol, low acid, no carbonyl
S6	Code 10	Base smoke: organic acid/carbonyls/phenols
S7	Code V	Organic acid preparation: low pH, medium acid, medium carbonyl, low phenol
S8	Cloud S-AC15	High buffered organic acid + medium carbonyl preparation

Table 3.3. Water activity (a_w) and pH of semi-moist pet food treatments without and with inclusion of liquid smoke (0.5% - 4%, w/w).

Treatment	**a_w**	**pH (0.5 - 4% smoke)**
Untreated	0.75 - 0.76	5.2 - 5.3
P-1720	0.75	4.6 - 5.3
Cloud S-5	0.75	4.9 - 5.2
Cloud S-C100	0.76 - 0.77	4.8 - 5.4
Black deli	0.75	4.9 - 5.3
Hickory OS-1473	0.75	4.6 - 5.3
Code 10	0.75	4.9 - 5.2
Code V	0.75	4.5 - 5.0
Cloud S-AC15	0.75	4.9 - 5.2

Table 3.4. Minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) of liquid smoke preparations (S1 to S8) in nutrient broth (PDB) against *Aspergillus flavus* and *Fusarium graminearum* in comparison with potassium sorbate, a known mold inhibitor. A positive control consisted of fungal inoculum only (no treatment), and a negative control consisted of potato dextrose broth (PDB) alone (no treatment, no fungal inoculation) (data not shown).

Treatment	***Aspergillus flavus***		***Fusarium graminearum***	
	MIC (%)[#]	**MFC (%)[#]**	**MIC (%)[#]**	**MFC (%)[#]**
P-1720	3.125	3.125	0.39	1.56
Cloud S-5	6.25	12.5	1.56	3.125
Cloud S-C100	1.56	3.125	0.39	0.39
Black deli	3.125	6.25	1.56	1.56
Hickory OS-1473	6.25	12.5	0.78	1.56
Code 10	1.56	6.25	0.39	1.56
Code V	3.125	6.25	0.78	1.56
Cloud S-AC15	1.56	3.125	0.39	0.39
Potassium sorbate	0.195	0.195	0.098	0.098

[#]The most recurring value of 3 replicates is reported as MIC or MFC.

Table 3.5. Mean number of days taken to observe wild-type mold growth in a model semi-moist pet food with inclusion of liquid smoke treatments (S1 to S8) at 0.5, 1, 2 and 4% in comparison with untreated (0% smoke). Potassium sorbate treatment (positive control) did not show visible mold growth during the 30-day period (not shown).

Treatment	**Number of days to mold (Mean ± SE)[abc]**				
	Concentration (%)				
	0.0	**0.5**	**1.0**	**2.0**	**4.0**
Untreated	7.7 ± 0.3 $_{c, A}$	-	-	-	-
P-1720		10.7 ± 0.7 $_{ab, A}$	11.7 ± 1.2 $_{ab, AB}$	14.5 ± 1.2 $_{ab, B}$	24.0 ± 0.0 $_{ab, C}$
Cloud S-5		12.0 ± 1.2 $_{b, AB}$	10.3 ± 0.7 $_{b, A}$	14.3 ± 0.7 $_{b, B}$	22.0 ± 1.6 $_{b, C}$
Cloud S-C100		13.3 ± 0.9 $_{a, A}$	15.0 ± 1.0 $_{a, AB}$	18.8 ± 0.8 $_{a, B}$	28.0 ± 0.0 $_{a, C}$
Black deli		10.7 ± 0.7 $_{ab, A}$	15.3 ± 2.4 $_{ab, B}$	19.7 ± 0.9 $_{ab, C}$	18.0 ± 0.0 $_{ab, BC}$
Hickory OS-1473		12.0 ± 2.1 $_{ab, A}$	13.0 ± 0.0 $_{ab, A}$	21.0 ± 1.5 $_{ab, B}$	25.0 ± 0.0 $_{ab, C}$
Code 10		13.3 ± 1.2 $_{ab, AB}$	11.0 ± 0.6 $_{ab, A}$	14.3 ± 1.2 $_{ab, B}$	17.0 ± 2.3 $_{ab, C}$
Code V		8.7 ± 0.3 $_{b, A}$	9.3 ± 0.3 $_{b, A}$	14.7 ± 1.5 $_{b, B}$	26.0 ± 1.0 $_{b, C}$
Cloud S-AC15		12.0 ± 1.0 $_{a, A}$	14.7 ± 1.8 $_{a, A}$	21.0 ± 0.8 $_{a, B}$	26.0 ± 0.8 $_{a, C}$

[a]Each mean is based on $n = 3$ replications.

[b]Means among the treatments within each concentration followed by different letters in lower case are significantly different ($P<0.05$, by Tukey' test).

[c]Within each treatment, means among concentrations followed by different letters in upper case are significantly different ($P<0.05$, by Tukey test).

Table 3.6. Mean logarithmic reduction (between day 0 and day 35) of *Aspergillus flavus* counts in semi-moist pet food treatments with inclusion of liquid smoke preparations (S1 to S8) at 0.5%, 1%, 2% and 4% concentrations in comparison with untreated (0% smoke) over 35 days incubation period. Initial load of *A. flavus* inoculated in the semi-moist pet food samples was 3.5 log CFU/mL. Potassium sorbate treatment (positive control) did not show mold growth during the 35-day period (not shown).

Treatment	Log reduction (Mean ± SE)[abcd]				
	Concentration (%)				
	0.0	0.5	1.0	2.0	4.0
Untreated	-1.3 ± 0.2 f, E	-	-	-	-
P-1720		-0.2 ± 0.4 e, B	-0.5 ± 0.4 e, BC	-0.6 ± 0.4 e, C	1.9 ± 0.3 e, A
Cloud S-5		-0.3 ± 0.3 e, B	-0.8 ± 0.4 e, C	-0.1 ± 0.2 e, B	1.6 ± 0.2 e, A
Cloud S-C100		-0.7 ± 0.2 b, C	0.6 ± 0.2 b, B	1.9 ± 0.1 b, B	2.5 ± 0.0 b, A
Black deli		-0.1 ± 0.3 bc, C	-0.2 ± 0.2 bc, C	0.2 ± 0.1 bc, B	2.4 ± 0.1 bc, A
Hickory OS-1473		-0.2 ± 0.3 cd, B	-0.3 ± 0.1 cd, B	-0.5 ± 0.3 cd, B	1.9 ± 0.3 cd, A
Code 10		-0.5 ± 0.2 b, D	0.0 ± 0.2 b, C	0.9 ± 0.2 b, B	2.4 ± 0.1 b, A
Code V		-0.4 ± 0.2 de, C	-0.1 ± 0.1 de, B	-0.3 ± 0.2 de, BC	1.5 ± 0.2 de, A
Cloud S-AC15		-0.4 ± 0.2 a, C	1.3 ± 0.4 a, B	1.8 ± 0.4 a, B	2.3 ± 0.2 a, A

[a]Negative values of log reduction indicate increase of mold counts over the 35-day period.

[b]Each mean is based on $n = 3$ replications.

[c]Means among the treatments at each concentration followed by different letters in lower case are significantly different ($P<0.05$, by Tukey test).

[d]Within each treatment, means among concentrations at each treatment followed by different letters in upper case are significantly different ($P<0.05$, by Tukey's test).

Table 3.7. Linear regression (linear model $y = a + bx$) parameters of logarithmic reduction of *Aspergillus flavus* counts in semi-moist pet food treatments with inclusion of liquid smoke preparations (S1 to S8) at 2% and 4% concentrations.

Treatment	**Concentration (%)**	**Linear regression parameters**			
		***a* ± SE**	***b* ± SE**	***R*²**	***D*-value[#] (days)**
P-1720	2.0	4.07	0.00	0.01	-
	4.0	3.74	-0.15	0.77	6.7
Cloud S-5	2.0	3.87	-0.02	0.59	50.0
	4.0	3.26	-0.09	0.63	11.1
Cloud S-C100	2.0	4.25	-0.12	0.75	8.3
	4.0	3.88	-0.20	0.87	5.0
Black deli	2.0	3.10	0.01	0.13	-
	4.0	3.37	-0.14	0.80	7.1
Hickory OS-1473	2.0	3.25	0.05	0.83	-
	4.0	2.90	-0.08	0.67	12.5
Code 10	2.0	3.47	-0.06	0.58	16.7
	4.0	3.63	-0.18	0.88	5.6
Code V	2.0	3.63	0.01	0.41	-
	4.0	2.93	-0.07	0.48	14.3
Cloud S-AC15	2.0	3.94	-0.11	0.83	9.1
	4.0	3.30	-0.13	0.84	7.7

a and *b* are linear regression parameters; *a* = intercept; *b* = slope.

[#]*D*-value (-1/*b*) shows the time in days for 1-log reduction of *A. flavus* counts. *D*-values were not calculated for treatments showing no log reduction of mold counts over time.

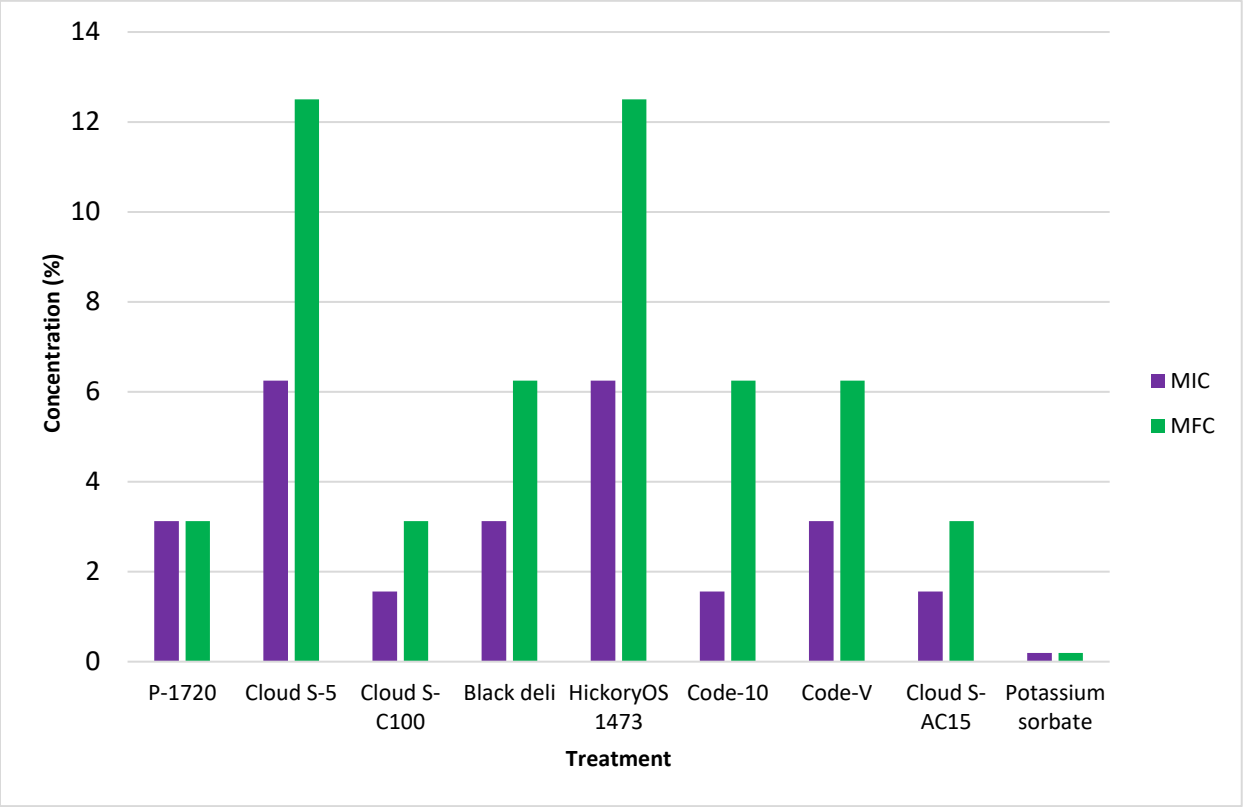


Fig. 3.1. Minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) of liquid smoke preparations (S1 to S8) in nutrient broth (PDB) against *Aspergillus flavus* in comparison with potassium sorbate, a known mold inhibitor. A positive control consisted of fungal inoculum only (no treatment), and a negative control consisted of potato dextrose broth (PDB) alone (no treatment, no fungal inoculation) (not shown).

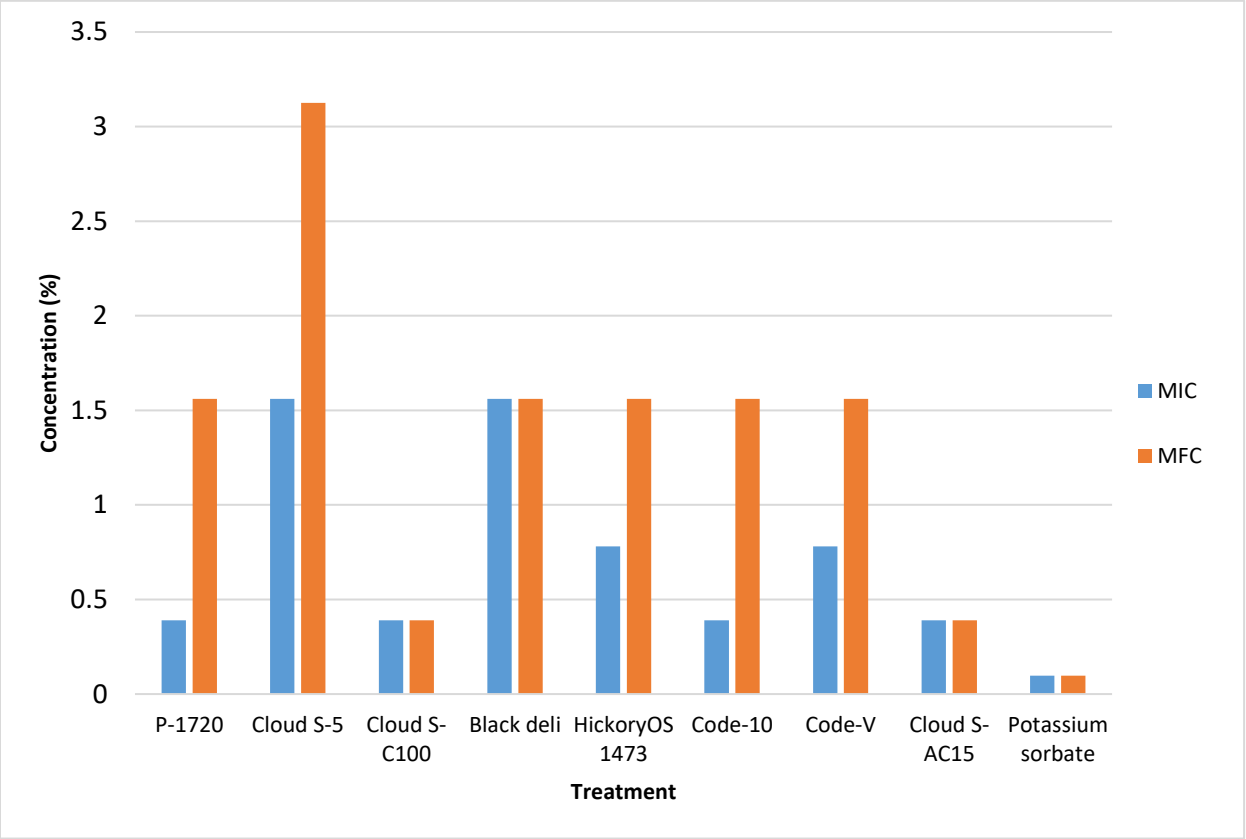


Fig. 3.2. Minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) of liquid smoke preparations (S1 to S8) in nutrient broth (PDB) against *Fusarium graminearum* in comparison to potassium sorbate, a known mold inhibitor. A positive control consisted of fungal inoculum only (no treatment), and a negative control consisted of potato dextrose broth (PDB) alone (no treatment, no fungal inoculation) (not shown).

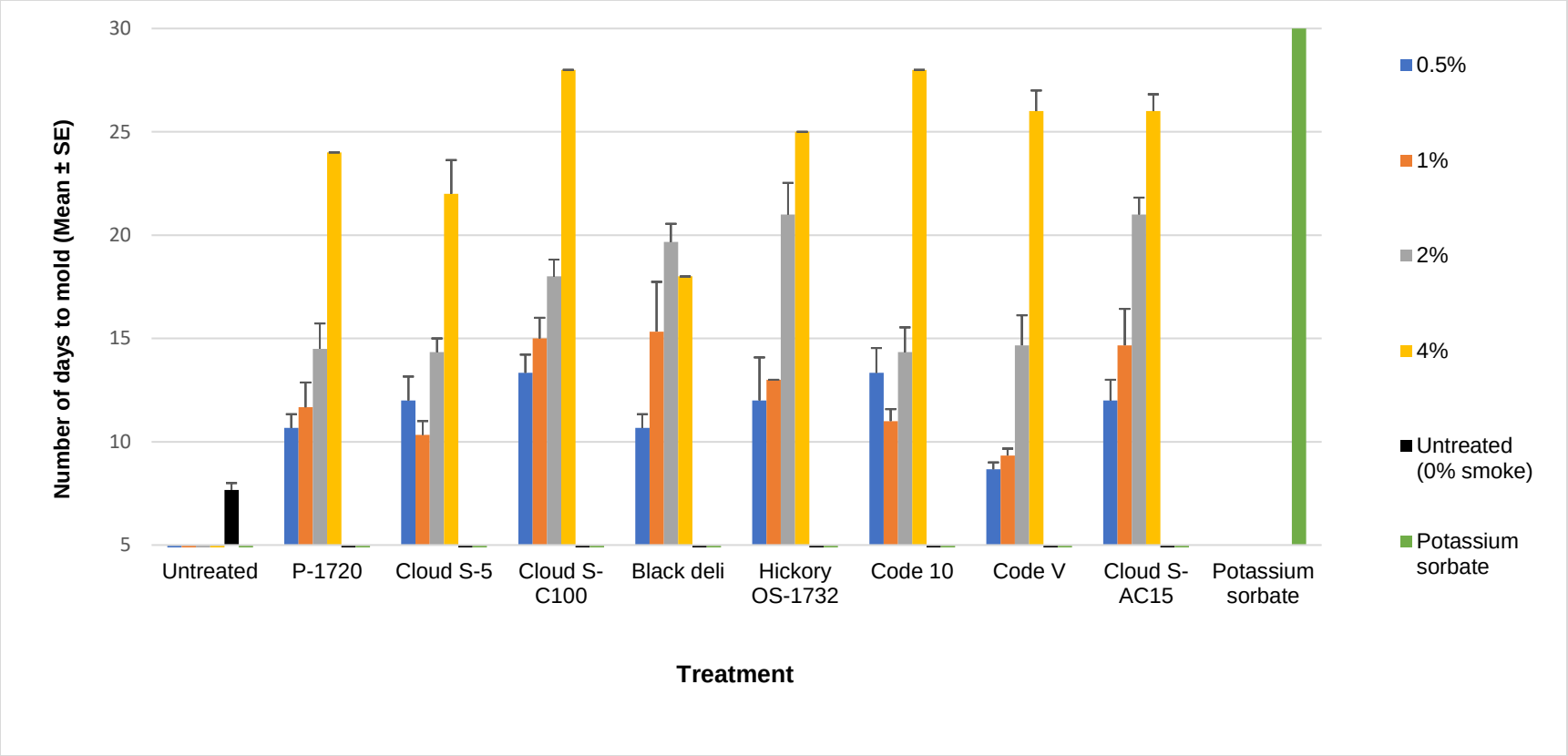
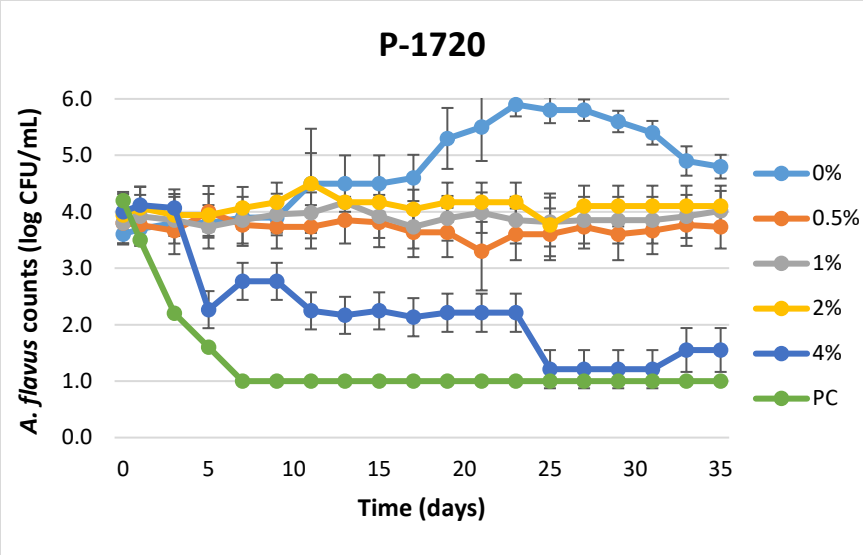
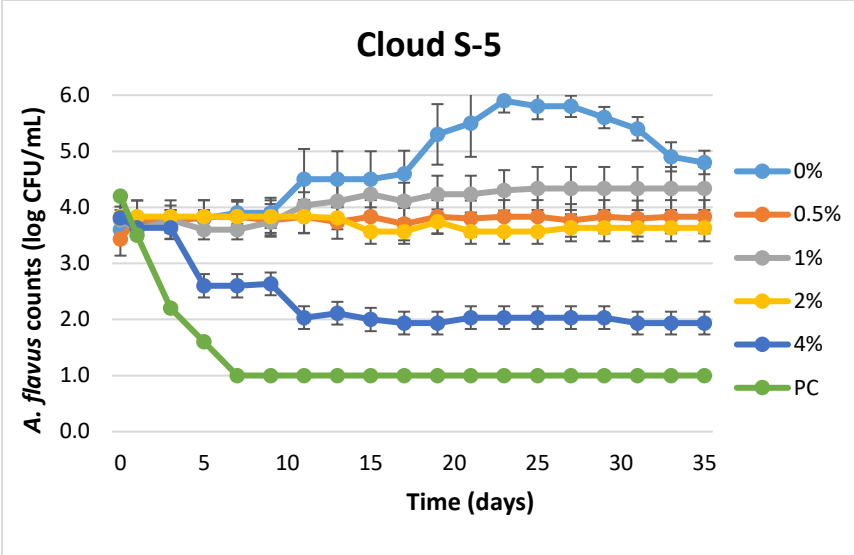
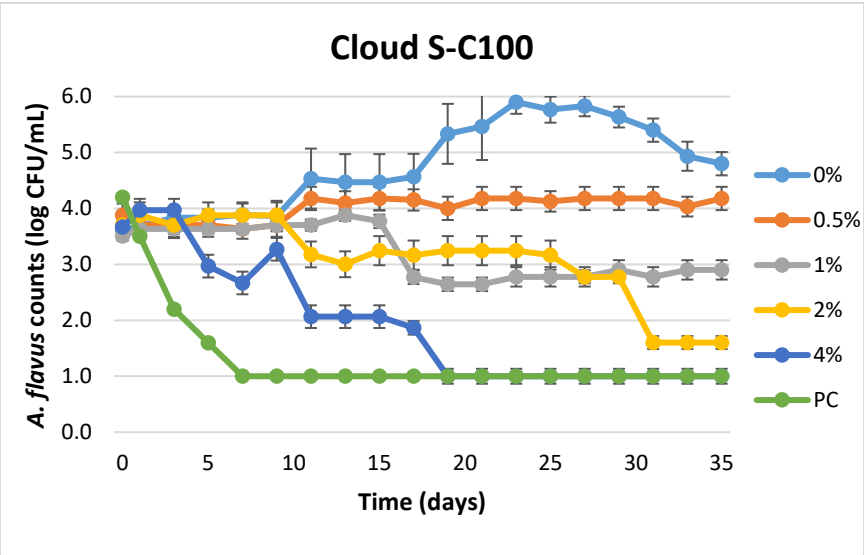
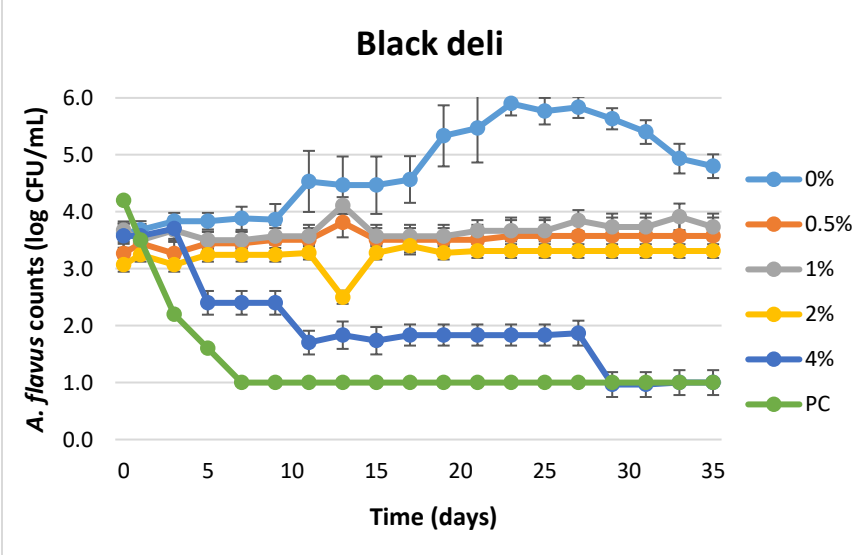


Fig. 3.3. Mean number of days taken to observe wild-type mold growth in a model semi-moist pet food with inclusion of liquid smoke treatments (S1 to S8) at 0.5%, 1%, 2% and 4% in comparison with untreated (0% smoke). Potassium sorbate treatment (positive control) did not show visible mold growth during the 30-day period.


P-1720
A. flavus counts (log CFU/mL)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
5
10
15
20
25
30
35
Time (days)
0%
0.5%
1%
2%
4%
PC


Cloud S-5
A. flavus counts (log CFU/mL)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
5
10
15
20
25
30
35
Time (days)
0%
0.5%
1%
2%
4%
PC


Cloud S-C100
A. flavus counts (log CFU/mL)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
5
10
15
20
25
30
35
Time (days)
0%
0.5%
1%
2%
4%
PC


Black deli
A. flavus counts (log CFU/mL)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
5
10
15
20
25
30
35
Time (days)
0%
0.5%
1%
2%
4%
PC

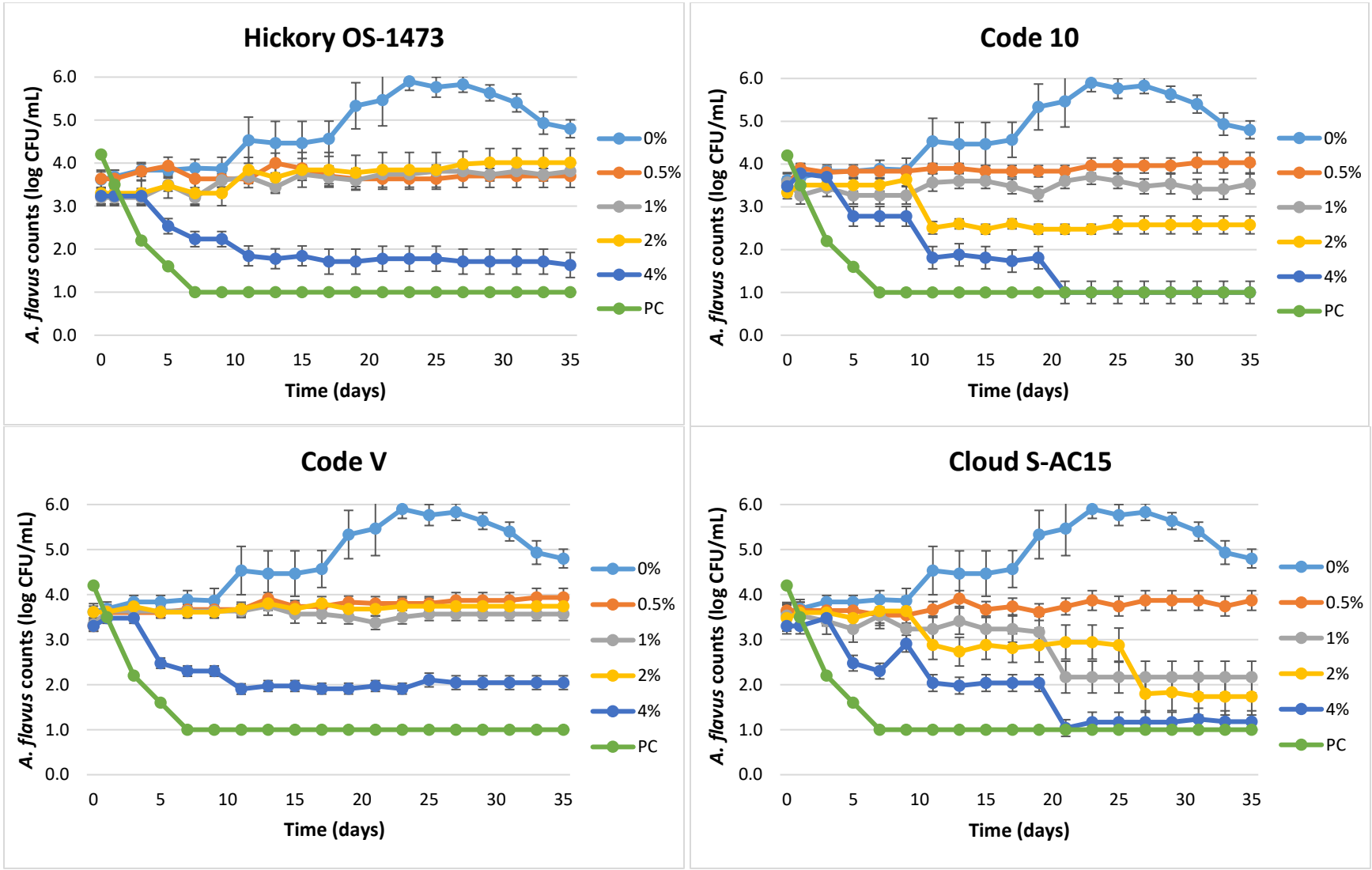


Fig. 3.4. Mean logarithmic counts (log CFU/mL) of *Aspergillus flavus* in semi-moist pet food treatments with inclusion of liquid smoke preparations (S1 to S8) at 0.5%, 1%, 2% and 4% concentrations in comparison with untreated (0% smoke). Potassium sorbate treatment (PC, positive control) did not show mold growth during the 35-day period. The detection limit is 1.0 log CFU/mL for sampling using PDA plates.

Chapter 4 - Use of organic acid mixtures to mitigate *Salmonella enterica*, *Escherichia coli* and *Aspergillus flavus* on dry pet food kibbles

Abstract

Post-processing operations of dry pet foods like extruded kibbles involve coating the product with fats and flavorings. These processes increase the risk for cross-contamination with foodborne pathogens such as *Salmonella* and *Escherichia coli* after the pasteurization stage. The heat utilized in the extrusion process to produce kibbles is sufficient to kill most enteric pathogens and yet recalls of pet foods in 2007 and 2012 were traced back to *Salmonella* contaminated kibbles. Recent studies from 2013 have shown that Shiga toxin-producing *E. coli* (STEC) could easily spread between humans and their pets. Mycotoxin contamination is also prevalent in pet foods due to presence of molds from raw materials and their proliferation during storage. In 2005, aflatoxicosis related illnesses in dogs and *Aspergillus flavus* contaminated dog food recalls were reported. Therefore, post-processing interventions to mitigate pathogenic bacteria and molds are needed to enhance the safety of dry pet foods. In this study, the antimicrobial effects of two types of organic acid mixtures - Activate DA™ and Activate WD-MAX™ against *Salmonella enterica, Escherichia coli* and *Aspergillus flavus* when used as a coating on dry pet food kibbles were evaluated. The minimum inhibitory concentration (MIC) of Activate DA (2-Hydroxy-4-methylthio-butanoic acid + fumaric acid + benzoic acid) and WD-MAX (2-Hydroxy-4-methylthio-butanoic acid + lactic acid + phosphoric acid) were determined using the broth micro- and macro-dilution assay technique. Using canola oil and dry dog digest as fat and flavor coating, the efficacy of Activate DA at 0%, 1% and 2%, and WD-MAX at 0%, 0.5% and 1% were tested on pet food kibbles inoculated with a cocktail of *S. enterica* serovars (Enteritidis, Heidelberg, and Typhimurium) or Shiga toxin-producing *E. coli* (STEC) serovars (O121, and O26) at 37°C for 0, 12, 24, 48, 72 hours, 30, and 60 days. Similarly, their efficacy was tested against *A. flavus* at 25°C for 0, 3, 7, 14, 21, 28, and 35 days. The MICs of Activate DA and WD-MAX were 1% and 0.5%

respectively, against the test organisms. Activate DA at 2% and Activate WD-MAX at 1% reduced (P<0.05) *Salmonella* counts by about 3 logs after 12 h and 4 - 4.6 logs after 24 h. Similarly, STEC counts were reduced by about 2 logs and 3 logs after 12 h and 24 h respectively. Levels of *A. flavus* did not vary (P>0.05) up to 7 days, and afterwards started to decline by >2 logs in 14 days, and up to 3.8 log reduction in 28 days for the dry and wet formula at 2% and 1%, respectively. The results suggest that the use of these organic acid mixtures during kibble coating may mitigate post-processing enteric pathogen and mold contamination on dry dog food kibbles, with Activate WD-MAX being effective at a lower concentration (0.5-1%) compared to Activate DA.

Key words: Pet food kibble, HMTBa, *Salmonella, E. coli,* STEC, *Aspergillus flavus,* antimicrobial

Introduction

A large percentage of pet owners prefer feeding dry commercial pet foods to their pets due to the convenience and nutritional benefits. Dry pet food constitutes the most commonly sold type of pet food in the world, forming 75.2% of dog food and 53.9% of cat food categories (Lange, 2016). These foods contain a variety of plant and animal ingredients/raw materials. The animal proteins are processed by different methods, predominantly in rendering plants, where there is a risk of contamination with various pathogens such as *Salmonella* (Vidyarthi et al., 2020). The gastro-intestinal tract of pets that consume these contaminated foods may get colonized with these bacteria, and yet they may not show any clinical symptoms (KuKanich, 2011). These occurrences confirm that pathogens like *Salmonella* can get transmitted to humans through handling of dry pet foods (Balachandran et al., 2012). Numerous cases of human salmonellosis have been linked to contaminated dry dog and cat food kibbles (KuKanich, 2011). Although these are extruded foods and go through a kill step during processing, they can get cross-contaminated with pathogenic bacteria during subsequent processing steps like coating with fats and flavors (Dhakal and Aldrich, 2020). By handling or consuming these foods, they may get transmitted to the pet which can serve as a carrier to humans. For instance, from 2006 to 2008, an outbreak of *Salmonella enterica* serotype Schwarzengrund, included 79 illnesses over 21 states, resulted in the recall of 105 brands of dry pet food and permanently closed a manufacturing plant in Pennsylvania (Behravesh et al., 2010). The process validation in the facility included a specified time-temperature combination to kill *Salmonella*, and then the food was subsequently moved to coating and flavoring steps where it was sprayed with fat and palatants. An epidemiological investigation led to the isolation of the bacterial strain related to the outbreak from the flavoring room, meaning that the *Salmonella* contamination occurred during that step (CDC, 2008). In 2012, there was also an outbreak of

human *Salmonella enterica* serotype Infantis infections related to exposure to dry dog food (CDC, 2012).

Escherichia coli are the most prevalent enteric bacteria in animals and humans, and are also an important zoonotic agent, which can be implicated in animal and human infectious diseases (Costa et al., 2008). A recent study showed that antibiotic-resistant *E. coli* could easily spread between humans and their pets (Leite-Martins et al., 2013) Shiga toxin-producing strains of *E. coli* O157:H7 have also caused numerous deaths following consumption of contaminated foods (Chahed et al., 2006). Many studies on the prevalence of antimicrobial resistance in *E. coli* isolates from farm animals and pets have been performed in other countries beyond the United States (Pedersen et al., 2007, Costa et al., 2008, Damborg et al., 2008). In 2016, the U.S. Food and Drug Administration (FDA) along with the Centers for Disease Control and Prevention (CDC) investigated a multi-state outbreak of Shiga toxin-producing *E. coli* (STEC) O121 and O26 infections. Sixty-three people infected with the outbreak strains of STEC O121 or O26 were reported from 24 states, and it was traced back to contaminated wheat flour from a General Mills facility in Kansas City, MO. Although pet food recalls due to STEC contamination have not been reported yet, recalls involving STEC-contaminated wheat flours have increased compared to previous years with 13 recalls occurring in 2019. Wheat flour is commonly used as an ingredient in pet foods, so the potential for STEC contamination exists.

Another risk factor for human and animal food safety is the presence of fungi and the potential for mycotoxins that they may produce. Hazardous mycotoxins can occur in cereal grains due to stress during toxigenic fungal growth, can be compounded with improper storage, and the process of cooking does not reduce their content (Cinar et al., 2019). During pet food manufacturing, the foods can be contaminated with mold spores especially when cereal grains are

ground, and the foods are pelleted or formed (Almeida et al., 2011). Dry pet food kibbles with water activity of 0.50 is not very conducive for mold growth, however mold spores prevalent in the environment can contaminate packaged foods that are opened by the consumer and can amplify during food storage, especially in a humid environment with >75% RH. Moldy foods reduce the nutritional value due to spoilage and under certain conditions may produce toxic metabolites called mycotoxins. These toxins with chemically diverse structures have been involved in disease outbreaks which have affected both animal and human health (Beardall et al., 1994; Prelusky et al., 1994). A study by Beuno et al. (2001) identified commonly occurring molds in 21 pet foods including dry dog kibbles across 8 commercial brands produced in Argentina, and were comprised mainly of *Aspergillus,* followed by *Rhizopus* and *Mucor* spp. Fungal contamination can lead to economic losses associated with nutrient and palatability reduction. Besides, the presence of mycotoxins also affects both animal and human's health (Savi et al., 2015). *Aspergillus flavus* being the most reported in pet food is responsible for the production of aflatoxins. Dogs are extremely sensitive to this group of toxins, with the liver being their main target (Silva et al., 2018). In the US in 2005, aflatoxicosis related illnesses in dogs and *A. flavus* contaminated dog food recalls were reported. More recently, in 2020, 28 deaths and 8 illnesses were reported in dogs that consumed the recalled Sportmix™ pet food product that was contaminated with aflatoxin. Pet food recalls by Sunshine Mills also happened in 2020, due to aflatoxin contamination from corn that was used as ingredient in the pet food (FDA, 2021). Thus, there is a need to control or reduce toxigenic A. *flavus* contamination.

The amino acid methionine is identified as a limiting amino acid in high forage cattle diets and has a positive impact on the health and performance of the animal. Methionine participates in a wide variety of metabolic pathways and serves as a precursor to other amino acids, like cysteine.

If free amino acids are fed directly to beef cattle, the rumen microbes destroy them before they even leave the rumen. Because amino acids must be presented to the small intestine in required amounts for the animal to synthesize protein, the amino acid must be protected or modified to avoid rumen degradation. Supplementing animal feed rations with a 'methionine hydroxy analogue' is an economical way to supply methionine. HMTBa (2-hyroxy 4-methlythio butanoic acid) is an organic acid and a methionine hydroxy analogue. It has been used as a methionine precursor in animal feed due to its unique chemical structure (Fig 4.1) that allows protection from some of the microbial degradation in the rumen gut. HMTBa also provides acidifying effects of organic acids. These acidifying effects subsequently provide gut health advantages to the animal by mitigating pathogen growth in the gut (Kaewtapee et al., 2010; Swennen et al., 2011). Methionine hydroxy analogue has also been shown to reduce nitrogen excretions (Kim et al., 2014), support animal performance during heat stress (Knight and Dibner, 1984; Dibner et al., 1992) and offer antioxidant capacity (Feng et al., 2011; Willemsen et al., 2011; Kuang et al., 2012; Li et al., 2014). Other than these health benefits and its use as a methionine precursor in animal feed supplements, its potential role in enhancing food safety of processed foods or its application in pet foods has not been investigated so far.

HMTBa is one of the main components of Activate DA™ and Activate WD-MAX™, which are proprietary blends of organic acids from Novus International (St. Charles, MO, USA). According to the company's product information, research conducted by the CCL Institute in the Netherlands using HMTBa demonstrated its effectiveness in reducing bacterial populations such as *Salmonella*, *E. coli* and *Campylobacter*, all of which can be found in the drinking water of poultry. The combination of organic acids in Activate DA and WD-MAX effectively reduces the pH of the gastrointestinal tract, promotes the establishment of a desirable and more balanced

intestinal flora and aids in digestion, providing more nutrients from feed and improving performance of the animal. Activate DA (HMTBa + fumaric acid + benzoic acid + silica + mineral oil) is a granular mixture applied to premixes and finished feeds. Activate WD-MAX (HMTBa + lactic acid + phosphoric acid) is used for acidification of drinking water for poultry, making the drinking water a less favorable environment for pathogen growth and it is shown to play an important role in the destruction of harmful microorganisms in the gut that could affect the birds' performance. Parker et al. (2007) evaluated the organic acid mixture Activate WD containing HMTBa at 0.04% and 0.08% in drinking water for poultry and found reduction in horizontal transmission of *Salmonella* in the broiler chickens. Guo-zheng et al. (2012) evaluated Activate WD against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella pullorum*, and *Campylobacter jejuni* in nutrient broth and found it to be effective at 0.6%. There is limited knowledge on the application of these organic acid mixtures containing HMTBa in dry pet food kibbles to enhance food safety.

The objectives of this research study were: (i) to determine the efficacy of organic acid mixtures, Activate DA and Activate WD-MAX, coated on extruded dry pet food kibble on the survival of *Salmonella enterica*, *Escherichia coli* and *Aspergillus flavus*; and (ii) to evaluate the residual antimicrobial effect of the organic acid mixtures Activate DA and Activate WD-MAX coated on extruded dry pet food kibbles on *Salmonella enterica*, to maintain *Salmonella*-free kibble despite repeated exposure to recontamination over time.

Materials & Methods

Manufacturing of dry pet food kibbles

Uncoated dry pet food kibbles were custom manufactured at Extru-Tech Inc. (Manhattan, KS, USA). The final moisture content of the kibble was 5.6% with water activity (a_w) of 0.50. The composition of the kibble is presented in Table 4.1.

Sources of organic acid mixtures

The organic acid mixtures evaluated in this study included Activate DA (dry formula) and Activate WD-MAX (liquid formula) that were provided by the study sponsor Novus International (St. Charles, MO, USA). Activate DA was a mixture of HMTBa, fumaric acid, benzoic acid, silica, and mineral oil. Activate WD-MAX was a mixture of HMTBa, lactic acid, and phosphoric acid. Activate DA which was in granular form was further ground to reduce its particle size using a laboratory hammer mill (Verder Scientific, Inc., Newtown, PA, USA) for use in the study.

Preparation of *Salmonella, Escherichia coli*, and *Aspergillus flavus* inoculum

Salmonella enterica serovars Enteritidis (ATCC 4931), Heidelberg (ATCC 8326), and Typhimurium (ATCC 14028) were procured from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in tryptic soy broth (TSB)-glycerol (7:3) at -80°C. Before use, the frozen cultures were streaked onto tryptic soy agar (TSA; BD Difco, Sparks, MD, USA) plates and incubated at 37°C for 24 h. A single colony of each *Salmonella* strain was inoculated into 10 mL of TSB (BD Difco, Sparks, MD, USA) and incubated at 37°C for 18 to 24 h. The cultures of each *Salmonella* serotype thus obtained were centrifuged for 10 min at 5000×g (Thermo Scientific, Waltham, MA, USA) at room temperature. The pellets were resuspended in pre-sterilized 0.1% peptone water (Difco Laboratories, Sparks, MD, USA), and an equal volume of each serotype was mixed to obtain the cocktail (~8 log CFU/mL).

Shiga toxin-producing *E. coli* (STEC) serovars O121 (ATCC 2219) and O26 (ATCC 2196) were maintained in tryptic soy broth (TSB)-glycerol (7:3) at -80°C. The same procedure explained earlier for the preparation of the *Salmonella* cocktail was followed for the STEC cocktail preparation. The final STEC cocktail inoculum had a concentration of ~8 log CFU/mL.

The fungal culture of *Aspergillus flavus* (ATCC 15548) was maintained in potato dextrose broth (PDB)-glycerol (7:3) at -80°C. Before use, the frozen cultures were streaked onto potato dextrose agar (PDA) with incubation at 25°C for 72 h. The fungal spores were collected from the grown culture on PDA by adding 5 mL of 0.1% peptone water to the surface of the dish. The spores were then dislodged from the solid medium using an L-shaped plastic rod. The spore suspension in peptone water was then collected and stored at 4°C and was used as the fungal inoculum (~4 log CFU/mL).

Experiment 1: Minimum inhibitory (MIC), bactericidal (MBC) and fungicidal concentrations (MFC)

The minimum inhibitory concentrations (MIC) of the organic acid mixtures were determined by the broth micro- and macro-dilution assay according to the antimicrobial susceptibility testing methods described by the Clinical and Laboratory Standards Institute (CLSI, 2014). To determine MIC, a 200 µL volume of organic acid mixture (Activate DA or WD-MAX) consisting of twice the desired final concentration were dispensed in the first well of a 96-well microtiter plate (triplicate wells) and 100 µL of sterile water in rest of the wells. A serial two-fold dilution of the organic acid mixtures was performed starting from 50% to 0.05% v/v concentration. A 100 µL aliquot of bacterial or fungal (*Salmonella* or STEC or *A. flavus*) culture (6 log CFU/mL for bacteria or 4 log CFU/mL for fungi) was added to each well of the plate already containing the 100 µL of decreasing concentrations of organic acid solutions to make a final volume of 200 µL

per well. A positive control consisted of bacterial or fungal inoculum only (no treatment), and a negative control consisted of tryptic soy broth (TSB) or potato dextrose broth (PDB) only. The MIC was determined as the lowest concentration of organic acid mixture that inhibited visible growth of the target microorganism in the microtiter plate after 24 h (for bacteria) or 72 h (for fungi) of incubation at 37ºC or 28ºC respectively.

To determine MBC and MFC, 100 µL of sample from each well from the MIC experiment was plated on to xylose lysine deoxycholate (XLD) agar for *Salmonella*, potato dextrose agar (PDA) for *A. flavus*, or 1 mL on to Petrifilm™ (3M, Minneapolis, MN, USA) plates for *E. coli* counts, for enumeration of *Salmonella*, *A. flavus* and STEC colonies at each concentration of organic acid mixture after incubation at 37ºC for 24 h (for *Salmonella*) or 37ºC for 48 h (for STEC) or 28ºC for 72 h (for *A. flavus*). The MBC and MFC were determined as the lowest concentration of organic acid mixture that caused absence (≤1 colony) of bacterial or fungal growth on the plates or Petrifilm. The study was replicated three times.

Experiment 2: *Salmonella, Escherichia coli* (STEC) and *Aspergillus flavus* mitigation on dry dog food kibbles coated with organic acid mixtures

A 180 g aliquot of dry pet food kibbles for each treatment was transferred to a plastic container and autoclave sterilized. For each of the organic acid treatments (Activate WD-MAX or DA), for each pathogen tested (*Salmonella,* STEC or *A. flavus*), a total of six containers were maintained: four for each of the treatment concentrations of organic acid mixtures (0.5% or 1% w/w of Activate WD-MAX, and 1% or 2% w/w of Activate DA), one for the untreated, and one for the negative control, for each pathogen tested. The organic acid mixtures were suspended in canola oil and applied to the kibbles using a pipette and mixing to coat them thoroughly to make a final weight of 200 g in each container. The final oil percentage on the kibbles was maintained

at ~7-8%. The concentrations of the organic acid mixtures used were based on the MICs (Table 4.2). The 'untreated' was without any organic acid treatment and inoculated with only the bacterial or fungal inoculum in TSB or PDB. The negative control was canola oil coated kibble, without any organic acid treatment and without inoculation with bacteria or fungi. After 30 min of the organic acid treatment (coating), a bulk-harvested *Salmonella* culture or STEC culture cocktail (~8 log) was spot inoculated on the kibbles using a pipette. The initial moisture content of the kibbles was 5.6% and the final moisture content post-inoculation was maintained at ~8-9% dry basis. For example, for 0.5% Activate WD-MAX treatment, 180 g kibbles + 14 g canola oil + 1 g organic acid (Activate WD-MAX) + 5 ml inoculum were used to make a total weight of 200 g in the container. After uniform mixing of the kibbles, the containers were incubated at 37°C. Similarly, for the fungal inoculation, *A. flavus* culture inoculum in PDB (~4 log) was spot inoculated on the kibbles. After uniform mixing of the kibbles, the containers were incubated at 28°C. Microbiological analyses for *Salmonella* and STEC were conducted for each of the containers at various predetermined time intervals: 2, 12, 24, 48, 72 hours, 30, and 60 days, and the fungal analyses for *A. flavus* were conducted at 1, 3, 7, 14, 21, 28, and 35 days. From each treatment, a 25 g subsample was collected in sterile Whirl-Pak bags (Nasco, Ft. Atkinson, WI) and was mixed in 225 mL of buffered peptone water (Difco Laboratories, Sparks, MD, USA) and stomached for 2 min. The mixtures were serially diluted in 0.1% peptone water and plated on XLD (for *Salmonella*), Petrifilm (for STEC) and PDA (for *A. flavus*). The XLD, PDA plates, and Petrifilms were incubated at 37°C, 25°C, and 37°C for 24 h, 72 h and 48 h respectively and then colonies were counted. The experiments were replicated three times.

Experiment 3: Residual antimicrobial effect of organic acid mixtures in dry pet food kibbles over time to mitigate exposure to *Salmonella*

Uncoated dry pet food kibbles as described earlier were used in this study. A *Salmonella* cocktail culture containing *Salmonella enterica* serovars Enteritidis (ATCC 4931), Heidelberg (ATCC 8326), and Typhimurium (ATCC 14028) was prepared as explained earlier and used as the inoculum (~8 log CFU/mL).

The minimum levels of organic acid mixtures Activate DA (1% and 2%) and WD-MAX at 0.5% and 1%) determined to be effective against *Salmonella* in pet food kibbles from experiment 2 were used in this experiment. For this experiment, *Salmonella*-negative kibble was coated with the minimum effective levels of organic acid mixtures Activate DA (1% and 2%) and WD-MAX (0.5% and 1%), divided into one of three challenge groups (for day-1, day-30 and day-90). Organic acid mixture-coated kibble was challenged with *Salmonella* after 1, 30 and 90 days of storage to investigate residual effect of the organic acid mixtures in the kibble on storage at 25°C. At each time period a freshly prepared *Salmonella* inoculum in TSB (as described earlier) was spot inoculated on the kibbles using a pipette and mixed thoroughly. The final moisture content of the kibbles post-inoculation was maintained at ~8-9% as described earlier. After the introduction of the challenge *Salmonella*, the kibble was incubated at 37°C for 24 h and then analyzed for *Salmonella* counts and enumerated on XLD agar plates as described in experiment 2. The untreated sample consisted of kibble with no organic acid coating and the negative control consisted of canola oil coated kibble (no organic acid and no *Salmonella* inoculation). The experiment was replicated three times.

Confirmative test for *Salmonella*

Confirmative test for *Salmonella* was conducted according to FDA-BAM method (Bacteriological Analytical Manual). In short, buffered peptone water from pre-enrichment of each

treatment sample, 1.0 mL and 0.1 mL, were transferred to 10 mL of Rappaport-Vassiliadis (RV; BD Difco, Sparks, MD, USA) and tetrathionate (TT; BD Difco, Sparks, MD, USA) broths, respectively, and incubated at 42°C for 24 h for selective enrichment of *Salmonella*. From each RV and TT broth tubes, one loopful was streaked onto xylose lysine deoxycholate (XLD) agar plates in duplicate. Inverted plates were incubated at 37°C for 24 h. Presumptive positive *Salmonella* colonies appeared as pink colonies with or without black centers, with most positive *Salmonella* producing colonies with large, glossy black centers or almost completely black. Presumptive *Salmonella*-positive colonies from XLD plates were then inoculated into triple sugar iron agar (TSI; BD Difco, Sparks, MD, USA) slants by streaking the slant and stabbing the butt and lysine iron agar (LIA; BD Difco, Sparks, MD, USA) slants by stabbing the butt twice and then streaking the slant. The TSI and LIA slants were incubated at 37°C for 24 h. Presumptive *Salmonella*-positive TSI reactions had alkaline (red) slants and acid (yellow) butts, while LIA reactions had alkaline (purple) butt with acidic (yellow) reaction negative for *Salmonella*. All cultures with an alkaline butt in LIA, regardless of TSI reaction, were retained as potential *Salmonella* isolates. Presumed-positive TSI and LIA slant cultures were inoculated into TSB and incubated at 37°C for 24 h, from which cells were harvested, DNA extracted and confirmed as *Salmonella* based on molecular analysis (Kim et al., 2006).

Statistical analysis

For the challenge study (experiment 2) for each of the three pathogens tested (*Salmonella*, STEC and *A. flavus*) the mean log reductions at each sampling time period for the treatments were subjected to two-way analysis of variance (ANOVA) using the GLIMMIX procedure of statistical software SAS (version 9.3) and the treatment means were separated using Tukey's post-hoc test when the *F*-test of the ANOVA per treatment was significant at $P<0.05$ (SAS Institute, 2011). The

linear model $y = a + bx$ was fit to logarithmic reduction of bacterial counts over time (hours) for the treatments, where *a* is the intercept and *b* is the slope. The decimal reduction time or *D*-value (time taken for 1-log reduction of bacterial counts) was calculated as the negative-inverse of slope (Mazzola et al., 2003).

Similarly, for experiment 3, the mean log reductions of *Salmonella* at each time period (day 1, day 30, day 90) for the treatments were subjected to two-way ANOVA and the treatment means were separated using Tukey's test ($P<0.05$) (SAS Institute, 2011).

Results

For experiment 1, the MICs and MBCs of Activate DA against *Salmonella* and STEC ranged from 0.5% to 1% and Activate WD-MAX ranged from 0.4% to 0.5%. The MIC and MFC of both Activate DA and WD-MAX against *A. flavus* was 2% (Table 4.2). Based on MIC, MBC and MFC results, the treatment concentration levels of Activate DA at 1% and 2% and Activate WD-MAX at 0.5% and 1% were used for experiments 2 and 3 subsequently for this study.

For experiment 2, the initial load of *Salmonella* in the inoculum was 8 log CFU/mL. A reduction in *Salmonella* counts ($P<0.05$) was observed over time (2 h to 60 days) due to inclusion of organic acid mixtures Activate DA and WD-MAX as coating on pet food kibbles as shown in Table 4.3 and Fig 4.2. The untreated (no organic acid coating) showed a constant mean *Salmonella* load of 6.9 log CFU/mL until 24 h. By 48 h the counts decreased to 4.7 log CFU/mL, by 72 h the counts further reduced to 1.7 log CFU/mL and by 60 days the counts declined to 1 log CFU/mL. On an average, a log reduction of 1.1 logs (from an initial load of 8 logs) was observed for the untreated samples until 24 h, 3.3 logs by 48 h, 6.3 logs by 72 h and 7 logs by day-60. Organic acid treatments Activate DA and WD-MAX reduced *Salmonella* counts over time ($P<0.05$) when compared to the untreated. Inclusion of organic acid mixture Activate DA at 1% decreased

Salmonella counts resulting in a population reduction of 5.1 logs (from an initial inoculum load of 8 logs) by 24 h and 7 logs by 48 h. Activate DA at 2% also followed a similar trend decreasing *Salmonella* counts by 5 logs at 24 h and 7 logs at 48 h. However, at 12 h Activate DA at 2% reduced *Salmonella* counts by 5.2 logs whereas at 1% the log reduction was only 4.2 logs at the same time point. Activate WD-MAX at 0.5% led to a log reduction of 6.5 logs at 24 h, and 7 logs at 48 h. A similar result was observed at 1% with a log reduction of 6.3 logs at 24 h and 7 logs at 48 h. At 12 h Activate WD-MAX at 1% reduced *Salmonella* counts by 5.4 logs whereas at 0.5% the log reduction was 4.3 logs at the same time point. Overall, there was no difference among the two organic acid treatments Activate DA and WD-MAX across the different concentrations tested. Considering the log reductions of *Salmonella* over time, Activate WD-MAX was effective at a lower concentration (0.5%) than Activate DA.

Similarly, a reduction in STEC counts was observed over time (2 h to 60 days) due to inclusion of Activate DA and WD-MAX as a coating on pet food kibbles (Table 4.4 and Fig 4.3). An average population reduction of 1.1 logs (from an initial load of 8 logs) was observed for the untreated samples until 24 h and then a reduction of 3.2 logs by 48 h, 5.4 logs by 72 h and 7 logs by day-60. Organic acid treatments Activate DA and WD-MAX reduced STEC counts over time ($P<0.05$) when compared to the untreated. Inclusion of Activate DA at 1% decreased STEC counts resulting in a population reduction of 5.1 logs (from an initial load of 8 logs) by 24 h and 5.7 logs by 48 h. Activate DA at 2% also followed a similar trend decreasing STEC counts by 5 logs at 24 h and 6.1 logs at 48 h. However, at 12 h Activate DA at 2% reduced STEC counts by 4.2 logs whereas at 1% the population reduction was only 3.2 logs at the same time point. Activate WD-MAX at 0.5% resulted in a reduction of 5.4 logs at 24 h followed by 6.5 logs at 48 h. At 1% also a similar trend was observed with a reduction of 5.3 logs at 24 h and 6.5 logs at 48 h. At 12 h

Activate WD-MAX at 1% reduced STEC counts by 4.4 logs whereas at 0.5% the log reduction was only 3.6 logs at the same time point. Overall, there was no difference among the two treatments Activate DA and WD-MAX across the different concentrations tested. Considering the log reductions over time, Activate WD-MAX was effective at a lower dose (0.5%) than the other treatments against STEC.

For the challenge study against *A. flavus*, log reductions were not observed consistently over time (1 to 35 days) with inclusion of Activate DA and WD-MAX as an oil-based coating on pet food kibbles (Table 4.5 and Fig 4.4). An average increase of 0.7 logs (from an initial load of 4 logs) was observed for the untreated samples through day 21 and then a reduction of 0.5 logs by day 35. Overall, organic acid treatments Activate DA and WD-MAX reduced *A. flavus* counts over time ($P<0.05$) when compared to the untreated. Inclusion of Activate DA at 1% decreased *A. flavus* counts by 0.4-0.9 logs by day 7 and 0.5 logs by day 35. Activate DA at 2% also decreased *A. flavus* counts by 0.9 logs at day 7 and 14, and 0.4 logs at day 35. Inclusion of Activate WD-MAX at 0.5% resulted in a reduction of 1.4 logs at day 7 followed by an increase of 0.5 logs at day 28 and then a 0.4 log reduction at day 35. At 1% Activate WD-MAX showed a reduction of 1.5 logs at day 7 followed by an increase of 0.6 logs at day 28 and then a 0.4 log reduction at day 35. Overall, there was no difference among the two organic acid treatments Activate DA and WD-MAX across the different concentrations tested against *A. flavus*. Consistent log reductions of *A. flavus* were not observed over time however the increase in mold counts was retarded due to the organic acid treatments compared to the untreated. Activate DA and WD-MAX exhibited a fungistatic effect during kibble storage but not a fungicidal effect.

The decimal reduction time known as *D*-value is the time (in hours) required to achieve 1-log reduction in bacterial counts. For *Salmonella*, Activate DA had *D*-values ranging from 1.01-

1.05 h (61-63 min) and Activate WD-MAX had *D*-values 1.03-1.08 h (62-65 min) (Table 4.6). However, there was no difference between the two treatments ($P>0.05$). Similarly, for STEC, Activate DA had *D*-values ranging from 0.98-1.02 h (59-62 min) and Activate WD-MAX had *D*-values 1.02-1.05 h (62-63 min), and there was no difference between the two treatments ($P>0.05$). *D*-values were not calculated for the effect of organic acid treatments against *A. flavus* as it did not show consistent log reduction of mold counts. Hence Activate WD-MAX at the lowest concentration of 0.5% can be considered effective against *Salmonella* and STEC on dry pet food kibbles.

For experiment 3 (Table 4.7 and Fig 4.5), the untreated (no organic acid) and treated (Activate DA and WD-MAX) pet food kibbles were inoculated with *Salmonella* (~8 log CFU/mL) on day-1, day-30 and day-90 to investigate the residual effect of the treatments over storage time. The untreated samples had ~7 log CFU/mL loads of *Salmonella* after inoculation on day-1, day-30 and day-90. Activate WD-MAX at 0.5% and 1% had residual effect in the pet food kibbles over time, and after inoculation on day-30 and analyzing it subsequently for *Salmonella* survival resulted in reductions of *Salmonella* by 4.3-5.4 logs. Similarly Activate DA at 1% and 2% had residual antibacterial effect in the pet food kibbles over time (on day-30) and resulted in reductions of *Salmonella* by 3.9-5.2 logs. Inoculating the treated kibbles with *Salmonella* on day-90 resulted in a similar decrease in bacterial counts by 4-5 logs for both Activate DA and WD-MAX. Compared to the untreated sample the organic acid treatments reduced *Salmonella* counts ($P<0.05$), however there was no difference between the two treatments Activate DA and WD-MAX ($P>0.05$) as shown in Table 4.7 and Fig 4.5. Therefore, both organic acid mixtures can be considered as having residual antibacterial effect for 90 days at concentrations 0.5-2% to mitigate repeated post-processing kibble exposure to *Salmonella*.

Discussion

The MIC, MBC and MFC tests are *in vitro* antimicrobial/antifungal susceptibility tests that are usually performed to evaluate the sensitivity of an organism to an antimicrobial or antifungal agent such as an antibiotic or chemical preservative. There have been only a few studies that investigated the antimicrobial properties of organic acid mixtures including HMTBa. Guo-zheng et al. (2012) evaluated Activate WD against *Staphylococcus aureus, Escherichia coli, Salmonella pullorum,* and *Campylobacter jejuni* and determined the minimum inhibitory concentration to be 0.3% and minimum bactericidal concentration to be 0.6%. Parker et al. (2007) evaluated Activate WD at 0.04% and 0.08% in drinking water for poultry and found reduction in horizontal transmission of *Salmonella* in the broiler chickens. These studies show that the organic acid mixtures containing HMTBa have potential antimicrobial effects in feed. From the MIC, MBC and MFC assays in this study (experiment 1), Activate DA (dry formula) and Activate WD-MAX (wet formula) were both found to be effective against *Salmonella,* STEC and *Aspergillus flavus* in nutrient broth (Table 4.2). From Table 4.2, Activate WD-MAX had consistently lower MIC, MBC and MFC results when compared to Activate DA, and hence is slightly more antibacterial against *Salmonella* and STEC and more antimycotic against *A. flavus* than Activate DA. Solubility of an antimicrobial/chemical in the MIC assay plays an important role in reacting with the target organism and its inhibition of growth in the assay. Activate DA is a dry powder which has some solubility issues. Our work was limited by a solubility of 1% in water at 25°C. There were concerns during the conduct of this research regarding the uniformity in water solution at the various concentrations tested for MIC. It was a powder with fine particle size after laboratory hammer milling to facilitate this study. The MIC, MBC and MFC of organic acid mixtures against microorganisms depend on several variables including composition and concentration of its

components, physical and chemical properties, and the culture conditions for the test microorganisms, and thus comparison of results with those of other studies involving organic acids is not simple. Iba et al. (1995) and Franco et al. (2005) reported that acidification of animal feeds by adding organic acids and organic salts can help control the growth of bacteria and fungi that reduce feed quality and produce toxins. The reduction of bacterial numbers in the animal's gut and improvement in the balance of gut microflora can potentially have an important prophylactic effect, reducing opportunity for infection associated with the proliferation of dangerous pathogens in the gut (Eidelsburger et al., 1992; Castro, 2005). Certain feed additive organic acid-mixtures include propionic, formic, and butyric acid which have been used in the past for their antimicrobial properties. These additives have previously been used as a method to control pathogens such as *Salmonella* and *E. coli* in poultry feed and other matrices (Van Immerseel et al., 2002; 2005). The organic acid mixtures evaluated in his study, Activate DA, is a mixture of HMTBa, fumaric acid and benzoic acid, and Activate WD-MAX is a mixture of HMTBa, lactic acid and phosphoric acid. This combination of acids should have an antimicrobial effect potentially, as the individual acids in these mixtures were already known to have preservative effects.

It is to be noted that the pH of Activate DA is slightly higher (pH 3) than Activate WD-MAX (pH 2) and so Activate WD-MAX has slightly better acidification property than Activate DA. Activate WD-MAX being a liquid formula is also very miscible in water at all concentrations. Comparing the composition of the mixtures tested, both primarily contain HMTBa but the secondary constituents differ: fumaric acid and benzoic acid for Activate DA, and lactic acid and phosphoric acid for Activate WD-MAX. Lactic and phosphoric acids have stronger acidification effects than fumaric acid, which also has solubility issues, and these could be the reasons why Activate WD-MAX has slightly better antimicrobial properties and hence lower MIC, MBC and

MFC than Activate DA. The MICs/MBCs of the organic acid mixtures were similar for *Salmonella* and STEC while their MIC/MFC against *A. flavus* was higher by 1%. This is because molds are more robust organisms than bacteria and *Aspergillus* species is found to be more tolerant to pH and fluctuations in growth conditions (Panwar et al., 2016; Casquete et al., 2017). So, these molds require greater antimicrobial effects possibly at a higher concentration of the antimicrobial to inhibit their growth. According to Pankey et al. (2004) when the ratio of MBC to MIC is less than or equal to 4, the antimicrobial can be considered bactericidal. Since the MBC and MFC to MIC ratios of organic acid mixtures in this study is in the range of 1 to 2, they can be considered as antimicrobial and should be effective against *Salmonella,* STEC and *A. flavus* when applied in food systems (Kiprotich et al., 2021).

The concentrations of antimicrobials tested effectively in nutrient broth may not be adequate to be tested in a food substrate. This is because several components in the food system like mineral salts can buffer the effects of antimicrobials. Therefore, it is common practice to test slightly higher concentrations of the antimicrobial in food systems. As the MIC/MBCs of Activate DA were 0.5-1%, we decided to test 1% and 2% of Activate DA for the food substrate kibble challenge study in experiment 2. Similarly Activate WD-MAX had MIC/MBCs of 0.4-0.5% in broth assay, so we tested it at 0.5% and 1% in kibbles for experiment 2.

Microbial challenge studies with *Salmonella*, STEC and *A. flavus* were conducted as experiment 2 in this study, with Activate DA and WD-MAX inclusion as coating on pet food kibbles at 1% and 2%, and 0.5% and 1% respectively, and enumerating the bacterial and mold counts over time (Fig 4.2, 4.3 and 4.4). For the *Salmonella* and STEC challenge studies, the initial load of bacteria inoculated to the kibbles was ~8 logs. For the untreated samples, while enumerating bacterial loads at the first time-point after inoculation i.e., 2 h, a reduction of about 1

log was observed (Tables 4.3 and 4.4). This was due to the limitation in recovery of bacterial loads from the food substrate during analysis. The bacterial loads remained consistent until about 48 h and then started to decline and decreased to about 1 log CFU/mL by 60 days. This was because kibbles are low water activity foods and incubation at 37°C over an extended period causes drying of the food substrate which inhibits bacterial growth by desiccation. It could also be due to chemical/enzymatic reaction kinetics at the different temperatures and differences in the physiological characteristics of the microbial cells at the different temperatures leading to inhibition of growth. At 2 h, the organic acid mixtures caused reductions of 2-2.5 logs for *Salmonella* and STEC, and by 12 h, the reductions ranged from 3.6-5.4 logs. By 24 h, in the treated kibbles, the organic acid mixtures reduced *Salmonella* loads by 5-6.5 logs and STEC loads by 5-5.4 logs. According to the US-FDA (United States Food and Drug Administration), the killing effect or antimicrobial activity of a substance needs to be ≥3 log reduction to be considered as an active ingredient. In our study about 2.5-5 log reductions were achieved in 12 h of treatment of the kibbles with the organic acid mixtures, which was promising. Use of multiple serovars of *Salmonella* and STEC as 'cocktail inoculums' in this study further corroborated the strong antimicrobial activity of the two organic acid mixtures.

For the *A. flavus* challenge study, pronounced log reductions of mold counts were not observed in the case of Activate DA and WD-MAX used as coating on pet food kibbles (Fig 4.3). While the untreated sample had a slight increase of mold counts by 0.5 logs by 28 days, the organic acid treated samples had a slight logarithmic decrease in mold counts by 1 log by 7 days and then an increase of 0.6 logs in mold counts. As the treated samples differed from the untreated ($P<0.05$) due to the fact that the increase in mold counts (as seen in the untreated samples) was slightly reduced by the presence of Activate DA and WD-MAX in the treated samples, we believe the

organic acid mixtures had a fungistatic effect on *A. flavus* in the kibbles. Another reason for the static log counts of *A. flavus* over the 35-day period could be because the blank kibbles used in this study already contained 2% vinegar (acetic acid) in its formula (Table 4.1). Vinegar is typically added as a clean-label preservative ingredient in pet foods for microbial shelf-life stability (Wall, 2019). It also acts as a mold inhibiting ingredient, similar to phosphoric acid which is used as a mold inhibitor in semi-moist pet foods. We propose conducting a mold challenge study with Activate DA and WD-MAX as coating on kibbles that were manufactured without inclusion of acids like vinegar or phosphoric acid to test the effects of these mixtures exclusively on *A. flavus*, with no interference or synergism from other acids. Also, in this challenge study, *A. flavus* counts on the untreated sample did not increase continuously during the 35-day incubation period. There was only a 0.5 log increase by day-35 despite no addition of organic acid mixtures. This was because pet food kibbles are low water activity foods (0.50 a_w) and incubation at 25°C for 35 days can result in drying of the food substrate and hence prevent mold count increase. Also, as it had been mold-challenged with 4 log inoculum there can be competition of nutrients for growth of mold colonies which slow their growth.

The *D*-values (Table 4.6) for *Salmonella* and STEC exposed to Activate DA and WD-MAX represent the times required for a 10-fold (90% or 1 log) destruction of the initial viable population of the pathogen. The linear regression model $y = a + bx$ was best fit for the treatments with higher R^2 values (>0.70) due to pronounced log reductions of the bacterial counts. The *D*-values for *Salmonella* and STEC were about 1 h in this study and there was no difference between the treatments Activate DA and WD-MAX (P>0.05). Because of the absence of fungicidal effect or a steady log reduction of mold counts in the case of *A. flavus* the linear model $y = a + bx$ was not fit to the regression over time and hence *D*-values were not calculated for *A. flavus*.

Residual antimicrobial effect of Activate DA and WD-MAX coated on pet food kibbles during a storage time from day-1 to day-90 was evaluated (Table 4.7, Fig 4.5) and the treated kibbles when exposed to *Salmonella* on day-30 resulted in a 3.9-5.4 log reduction of the bacteria. On day-90 also the *Salmonella* counts were reduced by 3-4.6 logs due to the organic acid treatment's residual effect during storage. Although not significant, the log reductions on day-90 were slightly lower when compared to day 30 probably because of the buffering effect of other ingredients in the kibble on the organic acids.

The mechanism of antibacterial activity of organic acids against gram negative bacteria like *Salmonella* has been described in previous research studies (Kashket, 1987; Russell, 1992; Hirshfield et al., 2003; Salsali et al., 2008). Organic acids in their undissociated and uncharged state are capable of bypassing bacterial cell membranes due to their lipophilic nature. Upon entering the more alkaline interior of a bacterium, the anion and proton from organic acids may have deleterious effects on the bacterium by increasing osmotic stress and disrupting important biomolecule synthesis, which finally causes bacterial death.

While the two organic acid mixtures Activate DA and Activate WD-MAX showed promising antibacterial properties against *Salmonella* and STEC when applied as a coating on pet food kibbles and have strong residual antimicrobial effect over extended storage times on the kibbles, their acceptability to pets (cats and dogs) need to be evaluated. Even though these have been in use as supplements in animal feed, we speculate that coating them on kibbles may have a strong effect of smell/flavor to the pets, which may lead to differences in acceptability. Dhakal and Aldrich (2020) reported that coating dog food kibbles with a combination of organic acids (medium chain fatty acids C8, C9 and C10) was effective in mitigation of *Salmonella* but reduced the acceptability of the kibbles due to strong aroma of the organic acids. Therefore, for this study

we propose conducting palatability tests, like a two-bowl forced choice evaluation test (Dhakal and Aldrich, 2020), to determine the acceptability of these organic acid coated kibbles by pets. In case of any changes in acceptability of these kibbles, a palatant (dog digest) may be necessary as a further coating to mask the aroma or flavor of the organic acid mixtures when applied to kibbles. Recently, combinations of organic acids and medium chain fatty acids (e.g., lauric acid) have also demonstrated synergistic benefits on animal intestinal health due to their antibacterial properties (Zentek et al., 2013). For future work we propose investigating synergistic effects of HMTBa with medium chain fatty acids as coating on pet food kibbles to control pathogen recontamination. Also, it is more likely for pathogens like *Salmonella* to be re-introduced to kibbles post processing via dust, flies or employee handling as opposed to a liquid contamination source. Therefore, we propose conducting challenge studies using a dry inoculum of *Salmonella* as water-based inoculum can have aversion to the oil-based surface coating on the kibbles.

Conclusion

In conclusion, the results of this study indicate that the use of organic acid mixtures Activate DA and WD-MAX as a coating ingredient on dry pet food kibbles showed a promising effect to mitigate post-processing *Salmonella* and STEC contamination. Being effective at low concentration among the treatments tested and for ease of application with no solubility issues we believe that Activate WD-MAX (wet formula) at 0.5% or 1% was the most effective to be used as a kibble coating to control *Salmonella* and STEC contamination.

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Tables & Figures

Table 4.1. Formulation of uncoated dry dog food kibbles.

Ingredient	Percent % w/w
Chicken meal	42.6
Corn meal	17.7
Wheat flour	17.7
Rice flour	17.7
Vinegar	2.0
Salt	0.5
Potassium chloride	0.3
Choline chloride	0.3
Dicalcium phosphate	0.3
Calcium carbonate	0.3
Trace mineral premix	0.2
Vitamin premix	0.1
Fish oil	0.1
Taurine	0.1
Natural antioxidant	0.1
Total	100.0

Table 4.2. Minimum inhibitory concentrations (MICs), minimum bactericidal concentrations (MBCs) and minimum fungicidal concentrations (MFCs) of organic acid mixtures Activate DA and Activate WD-MAX in nutrient broth (TSB or PDB) against *Salmonella, Escherichia coli* (STEC), and *Aspergillus flavus*. A positive control consisted of bacterial or fungal inoculum only (no treatment), and a negative control consisted of tryptic soy broth (TSB) or potato dextrose broth (PDB) only (no treatment, no inoculation) (data not shown).

Treatment	***Salmonella***		***E. coli* (STEC)**		***A. flavus***	
	MIC[#] (%)	**MBC[#] (%)**	**MIC[#] (%)**	**MBC[#] (%)**	**MIC[#] (%)**	**MFC[#] (%)**
Activate DA	0.5	1.0	1.0	1.0	2.0	2.0
Activate WD-MAX	0.4	0.5	0.4	0.5	1.0	1.0

[#]The most recurring value of the 3 replicates is reported as MIC, MBC or MFC.

Table 4.3. Mean logarithmic reduction of *Salmonella* counts in pet food kibble treatments with inclusion of organic acid mixtures Activate DA at 1% and 2% and Activate WD-MAX at 0.5% and 1% concentrations as coating, in comparison with untreated (0% organic acid). A negative control consisted of canola oil coated kibble (no organic acid, no inoculation).

Time	***Salmonella* log reduction (log CFU/mL) (Mean ± SE)[abc]**				
	Untreated	**Activate WD-MAX**		**Activate DA**	
	0.0%	**0.5%**	**1.0%**	**1.0%**	**2.0%**
0 h	0.0 ± 0.0 $_{a, A}$	0.0 ± 0.0 $_{b, A}$	0.0 ± 0.0 $_{b, A}$	0.0 ± 0.0 $_{b, A}$	0.0 ± 0.0 $_{b, A}$
2 h	1.1 ± 0.3 $_{a, B}$	2.2 ± 0.3 $_{b, B}$	2.2 ± 0.4 $_{b, B}$	2.2 ± 0.4 $_{b, B}$	2.2 ± 0.6 $_{b, B}$
12 h	1.1 ± 0.4 $_{a, C}$	4.3 ± 0.3 $_{b, C}$	5.4 ± 0.3 $_{b, C}$	4.2 ± 0.4 $_{b, C}$	5.2 ± 0.4 $_{b, C}$
24 h	1.1 ± 0.7 $_{a, C}$	6.5 ± 0.3 $_{b, C}$	6.3 ± 0.5 $_{b, C}$	5.1 ± 0.1 $_{b, C}$	5.0 ± 0.5 $_{b, C}$
48 h	3.3 ± 0.0 $_{a, D}$	7.0 ± 0.7 $_{b, D}$	7.0 ± 0.5 $_{b, D}$	7.0 ± 0.5 $_{b, D}$	7.0 ± 0.1 $_{b, D}$
72 h	6.3 ± 0.3 $_{a, D}$	7.0 ± 0.3 $_{b, D}$	7.0 ± 0.1 $_{b, D}$	7.0 ± 0.3 $_{b, D}$	7.0 ± 0.3 $_{b, D}$
30 d	7.0 ± 0.5 $_{a, D}$	7.0 ± 0.1 $_{b, D}$	7.0 ± 0.7 $_{b, D}$	7.0 ± 0.6 $_{b, D}$	7.0 ± 0.4 $_{b, D}$
60 d	7.0 ± 0.5 $_{a, D}$	7.0 ± 0.4 $_{b, D}$	7.0 ± 0.6 $_{b, D}$	7.0 ± 0.7 $_{b, D}$	7.0 ± 0.6 $_{b, D}$

[a]Each mean is based on $n = 3$ replications.

[b]Means among the treatments across concentrations followed by different letters in lower case are significantly different ($P<0.05$, Tukey's test).

[c]Within each treatment, means among different times followed by different letters in upper case are significantly different ($P<0.05$, Tukey's test).

Table 4.4. Mean logarithmic reduction of *E. coli* (STEC) counts in pet food kibble treatments with inclusion of organic acid mixtures Activate DA at 1% and 2% and Activate WD-MAX at 0.5% and 1% concentrations as coating, in comparison with untreated (0% organic acid). A negative control consisted of canola oil coated kibble (no organic acid, no inoculation).

Time	***E. coli* (STEC) log reduction (log CFU/mL) (Mean ± SE)[abc]**				
	Untreated	**Activate WD-MAX**		**Activate DA**	
	0.0%	**0.5%**	**1.0%**	**1.0%**	**2.0%**
0 h	0.0 ± 0.0 $_{a, A}$	0.0 ± 0.0 $_{b, A}$	0.0 ± 0.0 $_{b, A}$	0.0 ± 0.0 $_{b, A}$	0.0 ± 0.0 $_{b, A}$
2 h	1.1 ± 0.2 $_{a, B}$	2.6 ± 0.6 $_{b, B}$	2.7 ± 0.6 $_{b, B}$	2.1 ± 0.1 $_{b, B}$	2.2 ± 0.4 $_{b, B}$
12 h	1.1 ± 0.4 $_{a, C}$	3.6 ± 0.5 $_{b, C}$	4.4 ± 0.6 $_{b, C}$	3.2 ± 0.1 $_{b, C}$	4.2 ± 0.4 $_{b, C}$
24 h	1.3 ± 0.2 $_{a, D}$	5.4 ± 0.3 $_{b, D}$	5.3 ± 0.4 $_{b, D}$	5.1 ± 0.1 $_{b, D}$	5.0 ± 0.2 $_{b, D}$
48 h	3.2 ± 0.4 $_{a, E}$	6.5 ± 0.3 $_{b, E}$	6.5 ± 0.4 $_{b, E}$	5.7 ± 0.3 $_{b, E}$	6.1 ± 0.6 $_{b, E}$
72 h	5.4 ± 0.5 $_{a, F}$	7.0 ± 0.8 $_{b, F}$	7.0 ± 0.2 $_{b, F}$	7.0 ± 0.1 $_{b, F}$	7.0 ± 0.5 $_{b, F}$
30 d	7.0 ± 0.5 $_{a, F}$	7.0 ± 0.3 $_{b, F}$	7.0 ± 0.5 $_{b, F}$	7.0 ± 0.2 $_{b, F}$	7.0 ± 0.4 $_{b, F}$
60 d	7.0 ± 0.2 $_{a, F}$	7.0 ± 0.2 $_{b, F}$	7.0 ± 0.7 $_{b, F}$	7.0 ± 0.4 $_{b, F}$	7.0 ± 0.7 $_{b, F}$

[a]Each mean is based on $n = 3$ replications.

[b]Means among the treatments across concentrations followed by different letters in lower case are significantly different ($P<0.05$, Tukey's test).

[c]Within each treatment, means among different times followed by different letters in upper case are significantly different ($P<0.05$, Tukey's test).

Table 4.5. Mean logarithmic reduction of *Aspergillus flavus* counts in pet food kibble treatments with inclusion of organic acid mixtures Activate DA at 1% and 2% and Activate WD-MAX at 0.5% and 1% concentrations as coating, in comparison with untreated (0% organic acid). A negative control consisted of canola oil coated kibble (no organic acid, no inoculation).

Time	***A. flavus* log reduction (log CFU/mL) (Mean ± SE)[abc]**				
	Untreated	**Activate WD-MAX**		**Activate DA**	
	0.0%	**0.5%**	**1.0%**	**1.0%**	**2.0%**
0 h	0.0 ± 0.0 a, B	0.0 ± 0.0 b, B	0.0 ± 0.0 b, B	0.0 ± 0.0 b, B	0.0 ± 0.0 b, B
1 d	0.4 ± 0.1 a, C	0.4 ± 0.6 b, C	0.4 ± 0.4 b, C	0.4 ± 0.4 b, C	0.4 ± 0.2 b, C
3 d	0.5 ± 0.4 a, C	0.5 ± 0.4 b, C	0.4 ± 0.2 b, C	0.4 ± 0.2 b, C	0.4 ± 0.7 b, C
7 d	0.5 ± 0.6 a, D	1.4 ± 0.5 b, D	1.5 ± 0.1 b, D	0.9 ± 0.5 b, D	0.9 ± 0.6 b, D
14 d	-0.1 ± 0.7 a, C	0.6 ± 0.4 b, C	0.6 ± 0.3 b, C	0.6 ± 0.4 b, C	0.9 ± 0.4 b, C
21 d	-0.7 ± 0.3 a, A	-0.6 ± 0.6 b, A	-0.6 ± 0.3 b, A	-0.6 ± 0.7 b, A	-0.4 ± 0.3 b, A
28 d	-0.4 ± 0.2 a, B	-0.5 ± 0.7 b, B	-0.6 ± 0.5 b, B	0.4 ± 0.3 b, B	0.5 ± 0.6 b, B
35 d	0.5 ± 0.2 a, C	0.4 ± 0.8 b, C	0.5 ± 0.5 b, C	0.5 ± 0.4 b, C	0.4 ± 0.7 b, C

[a]Each mean is based on $n = 3$ replications.
[b]Means among the treatments across concentrations followed by different letters in lower case are significantly different ($P<0.05$, Tukey's test).
[c]Within each treatment, means among different times followed by different letters in upper case are significantly different ($P<0.05$, Tukey's test).

Table 4.6. Linear regression (linear model $y = a + bx$) parameters of logarithmic reduction of *Salmonella* and *E. coli* (STEC) counts in pet food kibble treatments with inclusion of organic acid mixtures Activate DA at 1% and 2% and Activate WD-MAX at 0.5% and 1% concentrations as coating.

Treatment	Concentration	Linear regression parameters			
	(%)	*a*	*b*	R^2	*D*-value[ab] (h)
***Salmonella**:*					
Activate WD-MAX	0.5	7.24	-0.97	0.77	1.03
	1.0	6.97	-0.93	0.73	1.08
Activate DA	1.0	7.50	-0.99	0.83	1.01
	2.0	7.22	-0.95	0.78	1.05
***E. coli* (STEC)**:*					
Activate WD-MAX	0.5	7.51	-0.98	0.86	1.02
	1.0	7.28	-0.95	0.84	1.05
Activate DA	1.0	7.93	-1.02	0.91	0.98
	2.0	7.58	-0.98	0.87	1.02

a and *b* are linear regression parameters; *a* = intercept; *b* = slope.
[a]*D*-value (-1/*b*) shows the decimal reduction time (in hours) for 1-log reduction of *Salmonella* and *E. coli* counts.
[b]*D*-values were not calculated for treatments showing no log reduction of *A. flavus* counts over time.

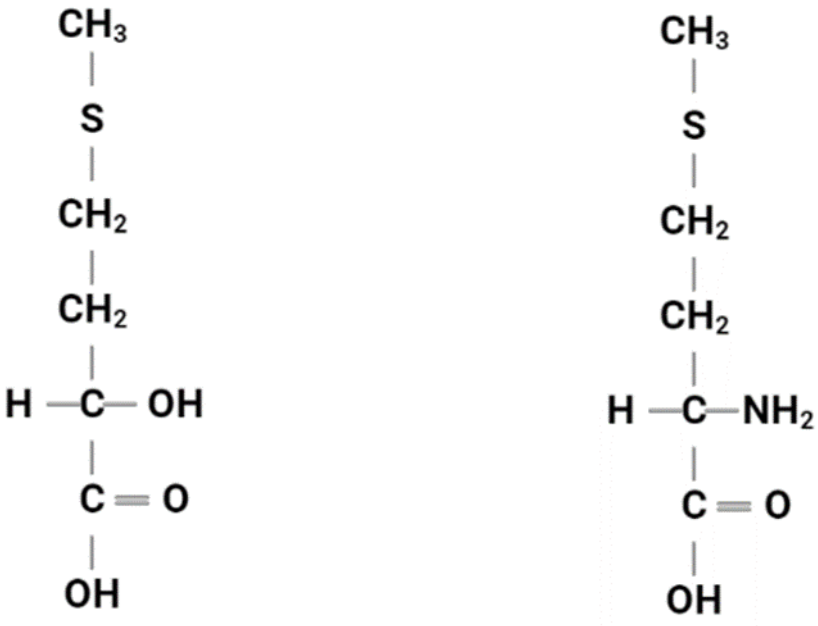
Table 4.7. Mean logarithmic reduction of *Salmonella* counts in pet food kibble treatments with inclusion of organic acid mixtures Activate DA at 1% and 2% and Activate WD-MAX at 0.5% and 1% concentrations as coating, in comparison with untreated (0% organic acid) showing residual effect of treatments over 1-, 30-, and 90-days during storage. A negative control consisted of canola oil coated kibble (no organic acid, no inoculation).

Time	***Salmonella* log reduction (log CFU/mL) (Mean ± SE)[abc]**				
	Untreated	**Activate WD-MAX**		**Activate DA**	
	0.0%	**0.5%**	**1.0%**	**1.0%**	**2.0%**
0 h	0.0 ± 0.0 $_{a, A}$	0.0 ± 0.0 $_{b, A}$	0.0 ± 0.0 $_{b, A}$	0.0 ± 0.0 $_{b, A}$	0.0 ± 0.0 $_{b, A}$
1 d	0.9 ± 0.8 $_{a, B}$	4.6 ± 0.7 $_{b, B}$	5.3 ± 0.7 $_{b, B}$	4.2 ± 0.9 $_{b, B}$	5.2 ± 0.5 $_{b, B}$
30 d	1.0 ± 0.9 $_{a, B}$	4.3 ± 1.0 $_{b, B}$	5.4 ± 0.5 $_{b, B}$	3.9 ± 0.4 $_{b, B}$	5.2 ± 0.7 $_{b, B}$
90 d	1.0 ± 0.8 $_{a, B}$	3.0 ± 0.6 $_{b, B}$	3.8 ± 0.4 $_{b, B}$	3.2 ± 0.7 $_{b, B}$	4.6 ± 0.4 $_{b, B}$

[a]Each mean is based on $n = 3$ replications.

[b]Means among the treatments across concentrations followed by different letters in lower case are significantly different ($P<0.05$, Tukey's test).

[c]Within each treatment, means among different times followed by different letters in upper case are significantly different ($P<0.05$, Tukey's test).



(a) HMTBa (b) Methionine

Fig 4.1. Comparison of chemical structures of HMTBa (methionine hydroxy analogue) vs. Methionine (amino acid).

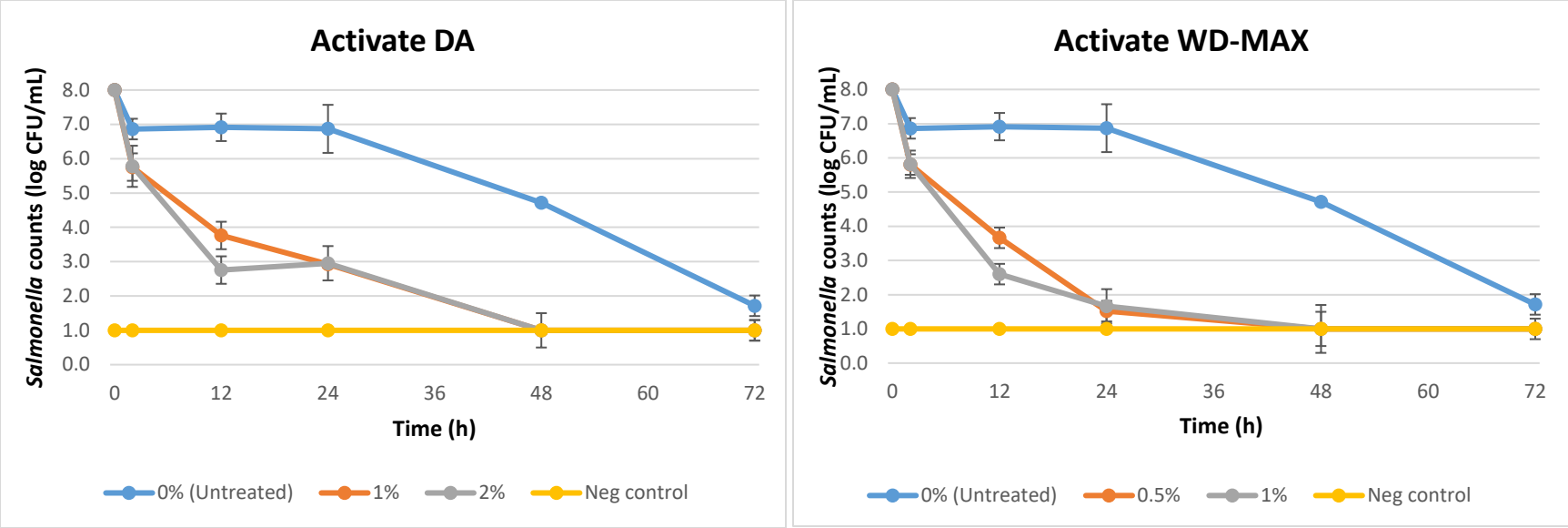


Fig. 4.2. Mean logarithmic counts (log CFU/mL) of *Salmonella* in pet food kibble treatments with inclusion of organic acid mixtures Activate DA at 1% and 2% and Activate WD-MAX at 0.5% and 1% concentrations as coating in comparison with untreated (0% organic acid). 0 h denotes initial load of *Salmonella* in inoculum before inoculation to the kibble. *Salmonella* counts at day-30 and day-60 are 1 log CFU/mL (not shown). A negative control consisted of canola oil coated kibble (no organic acid, no inoculation). Limit of detection is 1 log CFU/mL for this study.

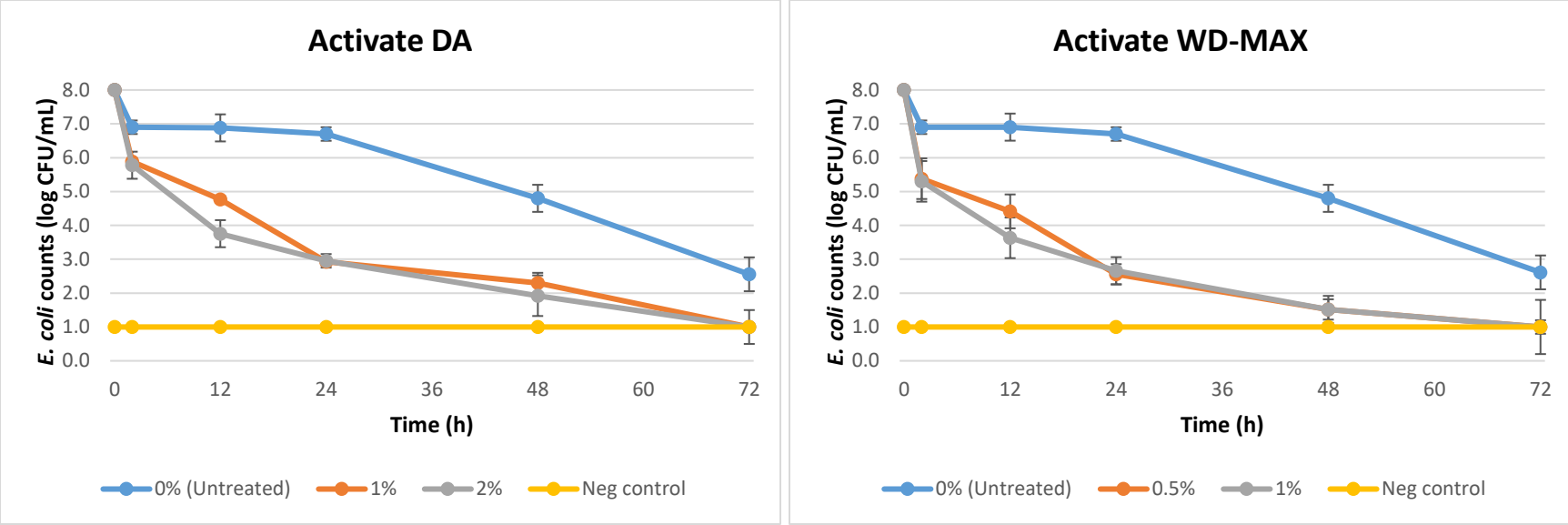


Fig. 4.3. Mean logarithmic counts (log CFU/mL) of *E. coli* (STEC) in pet food kibble treatments with inclusion of organic acid mixtures Activate DA at 1% and 2% and Activate WD-MAX at 0.5% and 1% concentrations as coating in comparison with untreated (0% organic acid). 0 h denotes initial load of *E. coli* in inoculum before inoculation to the kibble. *E. coli* counts at day-30 and day-60 are 1 log CFU/mL (not shown). A negative control consisted of canola oil coated kibble (no organic acid, no inoculation). Limit of detection is 1 log CFU/mL for this study.

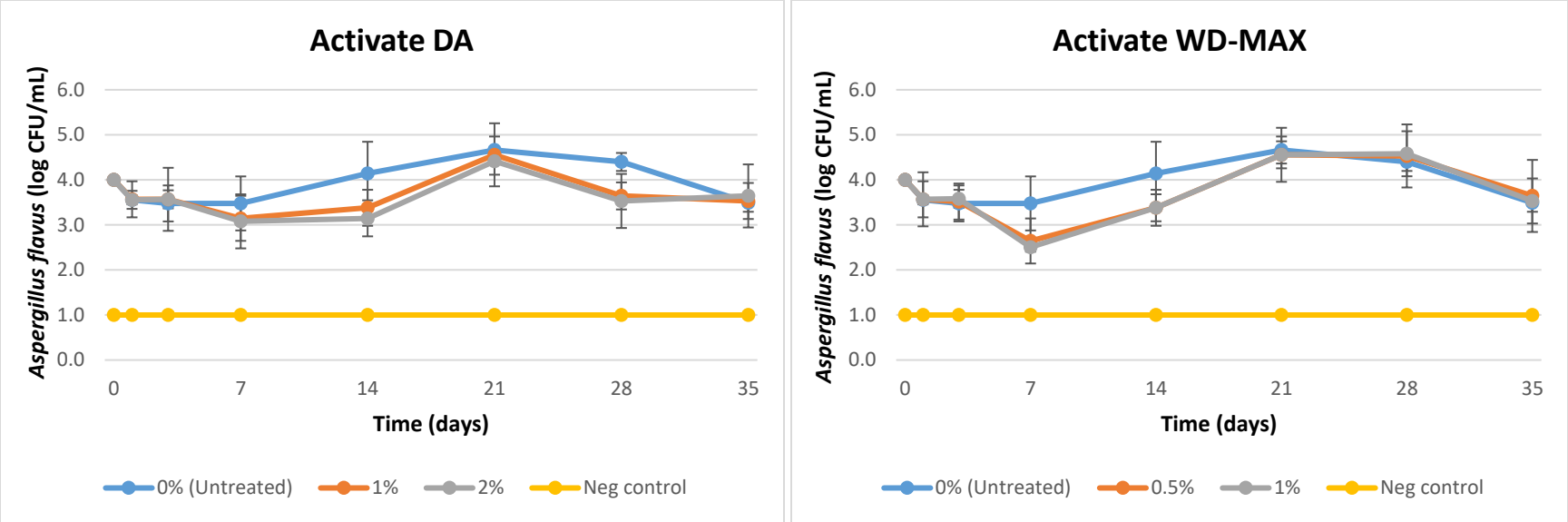


Fig. 4.4. Mean logarithmic counts (log CFU/mL) of *A. flavus* in pet food kibble treatments with inclusion of organic acid mixtures Activate DA at 1% and 2% and Activate WD-MAX at 0.5% and 1% concentrations as coating in comparison with untreated (0% organic acid). 0 h denotes initial load of *A. flavus* in inoculum before inoculation to the kibble. A negative control consisted of canola oil coated kibble (no organic acid, no inoculation). Limit of detection is 1 log CFU/mL for this study.

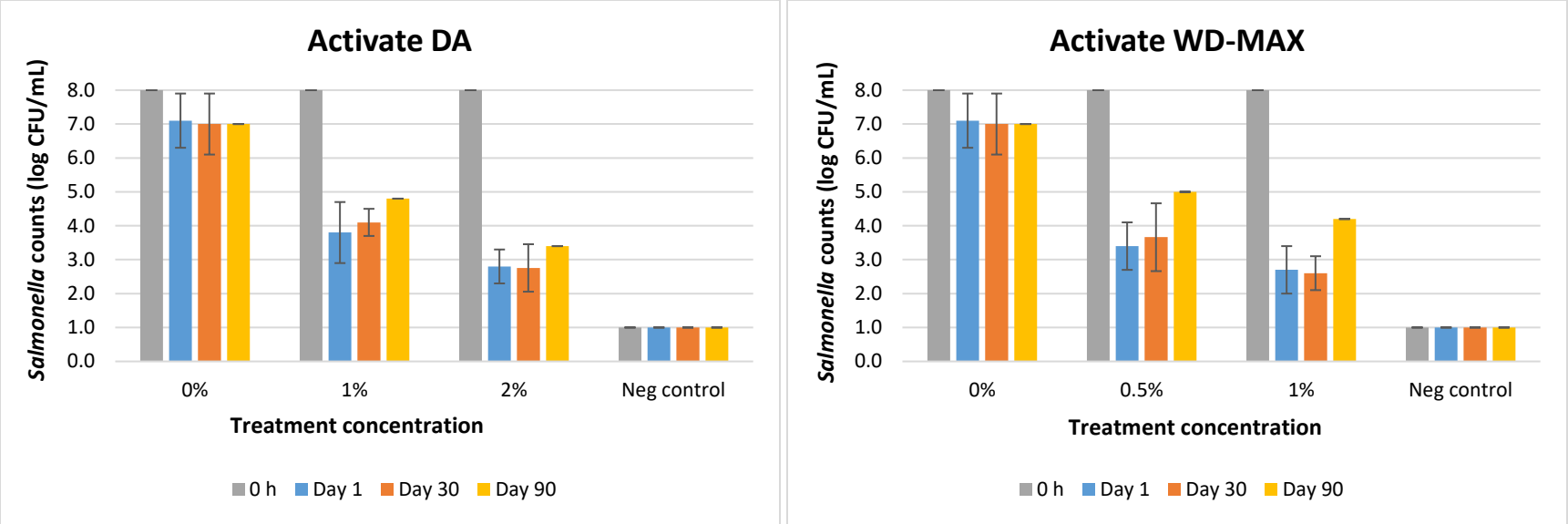


Fig. 4.5. Mean logarithmic counts of *Salmonella* in pet food kibble treatments with inclusion of organic acid mixtures Activate DA at 1% and 2% and Activate WD-MAX at 0.5% and 1% concentrations as coating, in comparison with untreated (0% organic acid) to investigate residual effect of treatments over 1, 30 and 90 days after repeated exposure to *Salmonella*. A negative control consisted of canola oil coated kibble (no organic acid, no inoculation). Limit of detection is 1 log CFU/mL for this study.

Chapter 5 - Use of organic acid mixtures to mitigate *Salmonella* on food contact surfaces

Abstract

Contaminated surfaces can transmit pathogens to food in industrial and domestic food handling environments. Exposure to pathogens on food contact surfaces may take place via cross contamination of pathogens during postprocessing activities. Formaldehyde-based commercial sanitizers in recent years are less commonly being used within food manufacturing facilities due to consumer perception and labeling concerns. There is interest to investigate clean-label, food-safe components for use on food contact surfaces to mitigate contamination from bacteria including *Salmonella*. In this study, the antimicrobial effects of two types of organic acid mixtures: Activate DA™ (2–Hydroxy-4-methylthio-butanoic acid [HMTBa] + fumaric acid + benzoic acid + silica + mineral oil) and Activate WD-MAX™ (HMTBa + lactic acid + phosphoric acid). Efficacy of Activate DA (1% and 2%) and Activate WD-MAX (0.5% and 1%) against *Salmonella* serovars (Enteritidis, Heidelberg, and Typhimurium) were evaluated on six different material surfaces - plastic (bucket elevator, tote bag), rubber (bucket elevator belt, automobile tire), stainless steel, and concrete. The untreated sample consisted of treatment with sterile water (no organic acids) and a positive control consisted of treatment with 30% formaldehyde. *Salmonella* counts were the highest (7.5-7.6 log CFU/cm^2) on stainless steel and plastic (bucket elevator) when compared to the other surfaces tested (rubber, plastic from tote and concrete) when no organic acid treatment was applied (untreated). There was a significant difference (P<0.05) on the log reduction of *Salmonella* on the material surfaces due to the organic acid treatments when compared to the untreated. The type of material surface also had an effect (P<0.05) on log reductions obtained. Stainless steel and plastic (tote) surfaces had the highest log reductions of *Salmonella* (3-3.5 logs), while plastic (bucket elevator) and rubber (tire) had the lowest log reductions (1-1.7 logs) after treatment with Activate WD-MAX. For Activate DA, lowest log reductions (~1.6 logs) of

Salmonella were observed for the plastic (bucket elevator) and rubber (tire), and the highest reductions were observed for plastic (tote), stainless steel and concrete (2.8-3.2 logs). Overall, the results suggested that Activate DA at 2% and WD-MAX at 1% were potentially effective at reducing *Salmonella* counts on food contact surfaces by 1.6-3.5 logs.

Key words: Stainless steel, rubber, plastic, sanitizer, HMTBa, *Salmonella*

Introduction

Foodborne pathogens cause millions of cases of sporadic illness and chronic complications, as well as large and challenging outbreaks in many countries. Surveys estimate that in the United States alone, bacterial enteric pathogens cause 9.4 million episodes of foodborne illness in humans, 55,961 hospitalizations, and 1,351 deaths each year (Heredia and Garcia, 2018). Salmonellosis caused by *Salmonella* Enteritidis is one of the most common foodborne diseases worldwide, accounting around 93.8 million foodborne illnesses and 155,000 deaths per year worldwide (Eng et al., 2015). Reports in the US account for more than one million people sickened by *Salmonella* each year (Heredia and Garcia, 2018). In the last decades regulations have moved in the direction of managing pet food safety at standards equivalent to human food. For instance, under the U.S. "Food, Drug, and Cosmetic Act", pet food should be "safe to eat, produced under sanitary conditions, contain no harmful substances, and be truthfully labeled" (U.S. Code, 2006), and pet food contaminated with *Salmonella* is considered adulterated (U.S. CFR, 2012). Facilities producing animal feed, including pet food, must register as human food facilities (FDA, 2006).

Contaminated surfaces can transmit pathogens to food in industrial and domestic food handling environments. Exposure to pathogens on surfaces may take place either directly by contact with contaminated objects or indirectly via aerosols originating from the surface. Various bacteria of public health significance, including *Salmonella*, *E. coli*, and *Listeria*, can survive on hands, sponges, clothes, utensils, and currency for hours or days (Scott and Bloomfield, 1990). Pathogenic bacteria may remain on equipment surfaces even after disinfection procedures are applied, increasing the risks associated with the transmission of diseases (Dunsmore et. al., 1981). Not only bacteria, but mold spores from the environment may also remain on equipment surfaces, on the floor of food manufacturing facilities, on automobile tires etc. and eventually find their way

to contaminate foods. Therefore, domestic and industrial food handling environments can be important sources of foodborne pathogens and spoilage organisms including bacteria and molds.

In a pet food manufacturing facility, batch-to-batch sequencing is used as a control measure to reduce hazard contamination (Schumacher et al., 2018). Though effective at reducing chemical hazards, it is found to be ineffective at reducing biological hazards which can occur through contaminated organic residue and dust remaining on surfaces of equipment and conveyors, as well as relatively large quantities of food product remaining in the boot of bucket elevator conveyors. In these instances, more strenuous physical cleaning may be necessary (Huss et al., 2015). However, the applicability of this option is limited because some of the equipment in pet food manufacturing facilities may not be designed for cleaning-in-place. Further complicating biological hazard control on these surfaces is the potential formation of biofilms. *Salmonella* have been shown to maintain presence on dry surfaces for up to 4 weeks through a biofilm (Habimana et al., 2010). Preventative measures utilized by food industry have included coating of surfaces to limit the establishment of vegetative cells or biofilms (Jullien et al., 2003). Huss et al. (2015) and Schumacher (2016) demonstrated that liquid decontamination of animal food manufacturing equipment that appears effective were not very practical or easy to implement. Generally, a water activity (a_w) level of 0.87 is required for growth of most bacterial pathogens of concern including *Salmonella*, so introducing a water-based sanitizer may raise the a_w to levels that allow for *Salmonella* growth. Hypochlorite and sodium chlorite can be effective detergents and sanitizers and are known to penetrate biofilms developed by *Salmonella*. Because of their potential impact on human health, chloride and its derivatives must be rinsed from surfaces prior to manufacturing food for consumption by pet animals or humans. Formaldehyde-based sanitizers also have bactericidal and fungicidal properties and are commonly used in animal production and health care

facilities. However, in recent years they have not been commonly used within food manufacturing facilities due to consumer perception and labeling concerns, in addition to potential carcinogenic effects to employees if handled improperly. Therefore, there is growing interest for clean-label, food-safe components to use on food contact surfaces that mitigate contamination from bacteria like *Salmonella*.

Supplementing animal feed rations with a 'methionine hydroxy analogue' is an economical way to supply methionine, a limiting amino acid in ruminants. HMTBa (2-hyroxy 4-methlythio butanoic acid) is an organic acid and a methionine hydroxy analogue. It has been used as a methionine precursor in animal feed due to its unique chemical structure (Fig 4.1) that allows protection from some of the microbial degradation of amino acids in the rumen gut. HMTBa also provides acidifying effects of organic acids. These acidifying effects subsequently provide gut health advantages to the animal by mitigating pathogen growth in the gut (Kaewtapee et al., 2010; Swennen et al., 2011). Methionine hydroxy analogue has also been shown to reduce nitrogen excretion (Kim et al., 2014), support animal performance during heat stress (Knight and Dibner, 1984; Dibner et al., 1992) and offer antioxidant capacity (Feng et al., 2011; Willemsen et al., 2011; Kuang et al., 2012; Li et al., 2014). Other than these health benefits and its use as a methionine precursor in animal feed supplements, its potential role in inhibiting microbial growth outside of the animal body, like on food contact surfaces, has not been investigated.

Research conducted by the CCL Institute in the Netherlands (Report CCL-FS/QS 04.0058) using HMTBa demonstrated its effectiveness in reducing bacterial populations such as *Salmonella*, *E. coli* and *Campylobacter*, all of which were found in the drinking water of poultry. HMTBa is one of the main components of Activate DA™ and Activate WD-MAX™, which are proprietary blends of organic acids from Novus International, Inc., the sponsor of this study. The combination

of organic acids effectively reduces the pH of the gastrointestinal tract, promotes the establishment of a desirable and more balanced intestinal flora and aids in digestion, providing more nutrients from feed and improving performance of the animal. Activate DA (HMTBa + fumaric acid + benzoic acid + silica + mineral oil) is a granular mixture applied to premixes and finished feeds. Activate WD-MAX (HMTBa + lactic acid + phosphoric acid) is used for acidification of drinking water for poultry, making the drinking water a less favorable environment for pathogen growth and it is shown to play an important role in the destruction of harmful microorganisms in the gut that could affect the birds' performance. There is limited knowledge on the application of these organic acid mixtures on foods contact surfaces as an antimicrobial agent to enhance food safety.

The objective of this study was to determine the efficacy of the organic acid mixtures, Activate DA and Activate WD-MAX, on food contact surfaces on the survival of *Salmonella.*

Materials & Methods

Sources of organic acid mixtures

The organic acid mixtures evaluated in this study were Activate DA (dry formula) and Activate WD-MAX (liquid formula) that were provided by the study sponsor Novus International (St. Charles, MO, USA). Activate DA was a mixture of HMTBa, fumaric acid, benzoic acid, silica, and mineral oil. Activate WD-MAX was a mixture of HMTBa, lactic acid, and phosphoric acid. Activate DA which was in granular form was further ground to reduce its particle size using a laboratory hammer mill (Verder Scientific, Inc., Newtown, PA, USA) for use in the study.

Preparation of *Salmonella* cocktail inoculum

Salmonella enterica serovars Enteritidis (ATCC 4931), Heidelberg (ATCC 8326), and Typhimurium (ATCC 14028) were procured from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were maintained in tryptic soy broth (TSB)-glycerol (7:3) at -80°C.

Before use, the frozen cultures were streaked onto tryptic soy agar (TSA; BD Difco, Sparks, MD, USA) plates and incubated at 37°C for 24 h. A single colony of each *Salmonella* strain was inoculated into 10 mL of TSB (BD Difco, Sparks, MD, USA) and incubated at 37°C for 18 to 24 h. The cultures of each *Salmonella* serotype thus obtained were centrifuged for 10 min at 5000×g (Thermo Scientific, Waltham, MA, USA) at room temperature. The pellets were resuspended in 0.1% presterilized peptone water (BD Difco, Sparks, MD, USA), and an equal volume of each serotype was mixed to obtain the cocktail (~8 log CFU/mL).

Contact surfaces and *Salmonella* inoculation

The following contact surfaces were used in this study: (i) plastic from bucket elevator, (ii) rubber from bucket elevator belt, (iii) rubber from automobile tire, (iv) plastic from polyethylene tote bag, (v) stainless steel, and (vi) concrete. The plastic from elevator bucket, rubber from elevator belt and stainless-steel surfaces were procured from used equipment from the Kansas State University Hal Ross flour mill. The concrete surfaces were made by producing poured QuickCrete™ in silicone molds, and the rubber from automobile tires were collected from used tires from a local automotive shop. Plastic from industrial tote bags was procured from Sterilite Inc. (Townsend, MA, USA). The materials were cut into square pieces measuring 4×4 cm.

Prior to the inoculation of the contact surfaces, each surface was soaked in 10% bleach solution for 15 min, followed by washing with detergent and rinsing thoroughly. After this they were immersed in ethanol for 15 min, followed by rinsing with sterile water and drying at 37°C. The surfaces were all transferred to sterile petri plates for inoculation with *Salmonella* and subsequent treatment with organic acid mixtures. For inoculation, 1 mL of *Salmonella*-cultured TSB was applied to each material surface and allowed to dry overnight at 37°C. To the inoculated surfaces, 1 mL of each treatment (1% or 2% of Activate DA, or 0.5% or 1% of Activate WD-

MAX) was applied and allowed to sit for 15 minutes. These concentrations of Activate DA and WD-MAX were used for evaluation in this study based on their minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) against *Salmonella* determined in Chapter 4 (Table 4.2) of this dissertation. For the untreated, each material surface was treated with sterile water instead of organic acids. For the positive control, the material surfaces were treated with 30% formaldehyde solution, which was the concentration of formaldehyde known to kill *Salmonella*, and commonly used in the commercial sanitizers like SalCurb® (Kemin Inc., Des Moines, IA, USA). Following treatment, the surfaces were vortexed in 9 mL of buffered peptone water for 5 mins for recovery of bacteria and then serially diluted in 0.1% peptone water and plated on xylose lysine deoxycholate (XLD). The plates were incubated at 37°C for 24 h and colonies were counted and expressed as log CFU/cm^2. All surface inoculations and subsequent treatments and enumeration were repeated for a total of 3 replicates.

Confirmative test for *Salmonella*

Confirmative test for *Salmonella* was conducted according to FDA-BAM method (Bacteriological Analytical Manual). In short, buffered peptone water (Difco Laboratories, Sparks, MD, USA) from pre-enrichment of each treatment sample, 1.0 mL and 0.1 mL, were transferred to 10 mL of Rappaport-Vassiliadis (RV; BD Difco, Sparks, MD, USA) and tetrathionate (TT; BD Difco, Sparks, MD, USA) broths, respectively, and incubated at 42°C for 24 h for selective enrichment of *Salmonella*. From each RV and TT broth tubes, one loopful was streaked to xylose lysine deoxycholate (XLD; BD Difco, Sparks, MD, USA) agar plates in duplicate. Inverted plates were incubated at 37°C for 24 h. Presumptive positive *Salmonella* colonies appeared as pink colonies with or without black centers, with most positive *Salmonella* producing colonies with large, glossy black centers or almost completely black. Presumptive *Salmonella*-positive colonies

from XLD plates were then inoculated into triple sugar iron agar (TSI; BD Difco, Sparks, MD, USA) slants by streaking the slant and stabbing the butt and lysine iron agar (LIA; BD Difco, Sparks, MD, USA) slants by stabbing the butt twice and then streaking the slant. The TSI and LIA slants were incubated at 37°C for 24 h. Presumptive *Salmonella*-positive TSI reactions had alkaline (red) slants and acid (yellow) butts, while LIA reactions had alkaline (purple) butt with acidic (yellow) reaction negative for *Salmonella*. All cultures with an alkaline butt in LIA, regardless of TSI reaction, were retained as potential *Salmonella* isolates. Presumed-positive TSI and LIA slant cultures were inoculated into TSB and incubated at 37°C for 24 h, from which cells were harvested, DNA extracted and confirmed as *Salmonella* based on molecular analysis (Kim et al., 2006).

Statistical analysis

The experiment was a 6×6 factorial arrangement of treatments using four organic acid treatment concentrations, one untreated, a positive control, and six material contact surfaces. The mean log reductions of *Salmonella* for the treatments were subjected to two-way analysis of variance (ANOVA) using the GLIMMIX procedure of statistical analysis software SAS (version 9.3) and the treatment means were separated using Tukey's post hoc test when the *F*-test of the ANOVA per treatment was significant at $P<0.05$ (SAS Institute, 2011).

Results

Logarithmic reduction in *Salmonella* counts was observed on the surfaces due to the treatment with organic acid mixtures Activate DA and WD-MAX (Table 5.1, Fig 5.1, and Fig 5.2). The initial load of *Salmonella* in the inoculum was 8 log CFU/mL. For the untreated surfaces, *Salmonella* counts were the highest on stainless steel at 7.6 log CFU/cm^2, followed by plastic (bucket elevator) which had 7.5 log CFU/cm^2 (Fig 5.1 and 5.2). *Salmonella* counts were the lowest on the plastic (tote) at 5.5 log CFU/cm^2. Concrete and rubber (tire, belt) had intermediate counts

(5.7-6.4 log CFU/cm^2). The positive control (30% formaldehyde) showed complete kill of *Salmonella*.

For both of the organic acid treatments (Activate DA and WD-MAX), plastic (bucket elevator) and rubber (tire) had the highest post-treatment *Salmonella* counts across all concentrations tested (6-7 log CFU/cm^2), and stainless steel and plastic (tote) had the lowest *Salmonella* recovered counts (4.5-5.6 log CFU/cm^2) (Fig 5.1 and Fig 5.2).

The untreated (no organic acid treatment) surfaces showed log reductions of *Salmonella* in the range of 0.4-2.5 logs even without organic acid treatment due to differences in bacteria attachment and recovery from the various material surfaces (Table 5.1). When compared to the untreated, the organic acid treatments had greater reductions of *Salmonella* counts (P<0.05). Across the various material surfaces, the treatment Activate WD-MAX at 0.5% had log reductions in the range of 1-3 logs, and at 1% had log reductions of 1.7-3.5 logs. Activate DA at 1% had log reductions of 1-2.8 logs, and at 2% had log reductions of 1.6-3.2 logs.

For Activate WD-MAX at 0.5%, the highest log reductions of 3 logs were observed for stainless steel followed by plastic (tote) and concrete. Plastic (bucket elevator) had the lowest log reduction (1 log) of *Salmonella,* followed by rubber (tire). For Activate WD-MAX at 1%, the highest log reduction of 3.5 logs was observed for stainless steel followed by plastic (tote) and concrete. The lowest log reduction (1.7 log) was observed for plastic (bucket elevator) followed by rubber (tire).

For 1% Activate DA, the highest log reductions (2.8 logs) were observed for plastic (tote) followed by concrete and rubber (belt), and the lowest log reduction (1 log) was observed for plastic (bucket elevator) followed by rubber (tire). For 2% Activate DA, the highest log reduction

of 3.2 logs was observed for stainless steel and plastic (tote), and the lowest log reduction (1.6 logs) was observed for plastic (bucket elevator) and rubber (tire).

Differences between treatment means within each treatment are reported in Table 5.1. There was no significant difference (P>0.05) between the two organic acid treatments Activate DA and WD-MAX on the log reduction of *Salmonella* on the contact surfaces.

Discussion

Mitigation of *Salmonella* in food manufacturing facilities include strategies like minimization of entry of the pathogen, point-in-time mitigation, and mitigation of post-processing contamination (Muckey, 2016). Food ingredients can be potential vectors of pathogenic bacteria, and the contaminated ingredients can contaminate facility equipment, leading to cross-contamination of other products. Thermal food processing techniques such as extrusion can reduce and/or eliminate pathogens in foods (Okelo et al., 2006; Cochrane et al., 2015). However, post-processing contamination with pathogens including *Salmonella* can occur during manufacturing, storage, and transportation of the finished food products through dust, air or employee handling which can lead to residual contamination in the finished product processing areas (Jones, 2011). To prevent the spread of such biological hazards, food manufacturing facilities have traditionally relied on good manufacturing practices (GMPs) and on the implementation of hazard analysis and critical control point (HACCP) plans. GMPs rely on facility design and layout that are amenable to effective physical cleaning, guidelines for employee hygiene and effective pest management to manage introduction of biological hazards into the facility (FAO, 2010). Many facilities rely on removal of food/ingredient residues and dust to manage further contamination, although physical cleaning usually is not an effective sanitation method (Huss et al., 2015). Huss et al. (2015) described that highly intensive liquid sanitation and heat was required to completely rid an animal

feed manufacturing facility from biological hazards. Therefore, it is safe to assume that food manufacturing equipment and contact surfaces may require substantial sanitization. Sanitization of surfaces can reduce post-processing cross-contamination and can be applied throughout the facility to decontaminate equipment if an undesirable microorganism like *Salmonella* has been established.

In this study two organic acid mixtures Activate DA (dry formula) and Activate WD-MAX (wet formula) were evaluated, which are food-safe, and were found to be effective in reducing *Salmonella* contamination on various surfaces namely plastic (bucket elevator, tote bag), rubber (bucket elevator belt, automobile tire), stainless steel, and concrete (Table 5.1). These material surfaces were chosen for evaluation as they encompass most of the different contact surfaces seen in an animal food manufacturing facility. For example, Davies and Wales (2010) found that dust, spillage, and aggregated materials at all stages of the food manufacturing operation including intake pits, ingredient silos, transfer augers and bucket elevators, weighing and mixing vessels, milling equipment, conditioners, pellet mills, coolers, finished product bins, and out-load gantries could serve as vectors of pathogen contamination. The floors of facilities are usually concrete which can harbor pathogens, products get transferred within the facility using plastic tote bags which can get cross contaminated with pathogens, and transport trucks that enter the facilities can bring in pathogens through the automobile tires.

In this study it was found that *Salmonella* counts were the highest (7.5-7.6 log CFU/cm^2) on stainless steel and plastic (bucket elevator) when compared to the other surfaces tested (rubber, plastic from tote and concrete) when no organic acid treatment was applied. These results were similar to the study by Muckey (2016) who reported that stainless steel and plastic were the most challenging surfaces to sanitize in an animal feed manufacturing facility, using liquid sanitizers.

The reason could be because the elevator bucket plastic surfaces that we used in our experiment were the ones that had wear and tear from use at the Hal Ross flour mill, KSU. When the surface of the plastic surface was not very smooth it could harbor growth of bacteria, and this aligns with practical situations where bucket elevators in food manufacturing facilities undergo wear and tear and when food residues get collected in the boot pit or bottom, it could propagate harboring and growth of pathogens like *Salmonella*. Similarly stainless steel is known to support pathogen growth of bacteria like *Salmonella* which can form biofilms as evaluated by previous research studies by Ronner et al. (1993), Shen et al. (2012), and Soni et al. (2013). This shows that plastic from bucket elevator and stainless steel can harbor *Salmonella* bacterial cells to a higher extent compared to other material surfaces.

The type of material surface had an effect ($P<0.05$) on log reductions obtained using the organic acid treatments (Table 5.1). On treatment with the wet organic mixture Activate WD-MAX, stainless steel and plastic (tote) surfaces had the highest log reductions of *Salmonella* (3-3.5 logs). This could be due to the smooth surface of stainless steel and plastic tote which helped in exposing the bacterial cells to the organic acids greatly. Plastic (bucket elevator) and rubber (tire) had the lowest log reductions of *Salmonella* (1-1.7 logs) after treating with Activate WD-MAX. Rubber (tire) is a corrugated surface and can have miniscule pores and tears, and elevator bucket plastic also due to wear and tear does not have a very smooth surface. Rubber (elevator belt) surface also has cracks and pits which can further develop additional surfaces to harbor bacteria, and previous research has demonstrated that rubber surfaces can resist sanitation by increased growth of bacteria like *Listeria* and *Salmonella* (Ronner and Wong, 1993). These could be reasons that Activate WD-MAX was least effective on these surfaces. Almost similar log reduction results were observed for treatment with the dry formula Activate DA, where the lowest

log reductions of *Salmonella* were observed for plastic (bucket elevator) and rubber (tire) (~1.6 log). The highest reductions were observed for plastic (tote), stainless steel and concrete (2.8-3.2 logs) probably due to their smooth surface as explained earlier. In our experiment we also found that stainless steel was more wettable with water than plastic (bucket elevator) and rubber (tire), which explains treatment with the organic acid mixtures more effective on stainless steel surface than the others.

This study indicates that both Activate DA and WD-MAX were effective at reducing *Salmonella* counts on food contact surfaces by 1-3.5 logs, with the higher concentrations tested (Activate WD-MAX at 1% and DA at 2%) causing reductions of 1.6-3.5 logs. According to the United States Environmental Protection Agency (US-EPA) a sanitizer should demonstrate at least a 3-log reduction (99.9%) of the test microorganism (a gram-positive and a gram-negative organism) in 5 minutes on non-food contact surfaces (e.g. rubber tire) to be effective, while it should be at least 5-log reduction (99.999%) in 30 seconds for food contact surfaces (e.g. stainless steel). In our study about 2.8-3.5 log reduction was achieved in the case of stainless steel, plastic (tote) and concrete due to treatment with the organic acid mixtures Activate DA and WD-MAX at 2% and 1% respectively.

There have been previous research studies evaluating efficacy of natural/food-safe components as sanitizers on food contact surfaces to mitigate pathogens including *Salmonella* (Soni et al., 2012; Desai et al., 2012; Nostro et al., 2007). However, these sanitizers being plant essential oils were not economically feasible for application in a food manufacturing facility. Whereas Activate DA and WD-MAX were not as expensive as plant essential oils and hence be potentially feasible to apply as a sanitizer or antimicrobial in an industrial setting at optimum concentrations of 1-2%.

The mechanism of antibacterial activity of organic acids against gram negative bacteria like *Salmonella* had been described in previous research studies (Kashket, 1987; Russell, 1992; Hirshfield et al., 2003; Salsali et al., 2008). Organic acids in their undissociated and uncharged state are capable of bypassing bacterial cell membranes due to their lipophilic nature. Upon entering the more alkaline interior of a bacterium, the anion and proton from organic acids may have deleterious effects on the bacterium by increasing osmotic stress and disrupting important biomolecule synthesis, which finally causes bacterial death.

Activate DA which is a dry powdered formula was evaluated in this study at 1% and 2% by dissolving in water w/w. We did not evaluate it as a dry sanitizer due to difficulty in uniform application on the surface pieces, as dry application would need special applicator/equipment for uniform dusting at low or optimal amounts both in laboratory and in a real food manufacturing facility. However, Muckey (2016) reported that dry sanitizers have an advantage over wet sanitizers as the wet sanitizers would require rinsing with water and complete drying prior to later manufacturing in the facility. Muckey (2016) evaluated sodium bisulfate (a dry acidulant) as a dry sanitizer on food contact surfaces and obtained 2.7 log reduction of *Salmonella* on stainless steel. From this study we propose evaluating Activate DA too as a dry sanitizer on food contact surfaces so that it could potentially be used in facilities handling dry-bulk systems. Muckey (2016) also evaluated a commercial food grade sanitizer and obtained 1.9 log reduction on stainless steel. Compared to it, the food grade organic acid mixtures tested in our study, Activate DA and WD-MAX, showed a higher log reduction of 2.8-3.5 logs on stainless steel against *Salmonella*.

It was reported in the previous chapter (Chapter 4) of this dissertation that Activate DA and WD-MAX had effective antimicrobial activity when applied as a coating on pet food kibbles at 0.5-2%. This property of inclusion of these organic acid mixtures in the food that is

manufactured can also act as a potential sanitizing agent for the food contact surfaces, as for example, when the organic acid treated food kibbles pass through a conveyor, they can have a potential antimicrobial activity on the surface of the conveyor where they come in contact over an extended period of time. This method of antimicrobial activity warrants validation.

Corrosiveness of the organic acid mixtures evaluated in this experiment were not measured and was outside the scope of this experiment. However, it is an important aspect to consider when evaluating sanitizers and so we propose testing for equipment corrosiveness using Activate DA and WD-MAX in future experiments.

Conclusion

In summary, surfaces and equipment in food manufacturing facilities can get contaminated with pathogens like *Salmonella* via post-processing cross-contamination, with plastic and stainless-steel surfaces being more susceptible in harboring and sustaining growth of *Salmonella*. Evaluation and selection of a sanitizer should consider microbial efficacy, practicality of application, application time, impact of surface type on effectiveness and corrosiveness, and cost (Marriott and Gravani, 2006). Prior to applying a sanitizer treatment to any surfaces, cleaning is necessary to reduce surface tension and remove organic material. Effective cleaning, which may require both physical cleaning and the use of cleaning solutions, removes biofilm formations that will allow for subsequent penetration and removal of vegetative bacteria by a sanitizer. Inadequate removal of organic matter during physical cleaning can provide adequate conditions for bacterial growth, increase cross-contamination and reduce sanitizer efficacy (Huss et al., 2015). From this study it can be considered that Activate WD-MAX at 1% and Activate DA at 2% can be effective sanitizers on food contact surfaces including steel, rubber and plastic. While this study yielded valuable data as a starting point to identify Activate DA and WD-MAX as potentially effective

sanitizers, additional research is warranted to determine practical dosages and application methods based on feasibility in an industrial setting.

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Tables & Figures

Table 5.1. Mean logarithmic reduction (log CFU/cm^2) of *Salmonella* counts in pet food contact surfaces treated with organic acid mixtures Activate DA at 1% and 2% and Activate WD-MAX at 0.5% and 1% concentrations in comparison with untreated (0% organic acid). Formaldehyde 30% treatment (positive control) had complete kill of *Salmonella* counts in 24 h (not shown).

Contact surface	***Salmonella* log reduction (log CFU/cm^2) (Mean ± SE)[abc]**				
	Untreated	**Activate WD-MAX**		**Activate DA**	
	0.0%	**0.5%**	**1.0%**	**1.0%**	**2.0%**
Stainless steel	0.4 ± 0.1 $_{a, B}$	3.0 ± 0.4 $_{b, B}$	3.5 ± 0.4 $_{b, B}$	2.4 ± 0.2 $_{b, B}$	3.2 ± 0.1 $_{b, B}$
Plastic (Bucket elevator)	0.5 ± 0.1 $_{a, D}$	1.0 ± 0.3 $_{b, D}$	1.7 ± 0.3 $_{b, D}$	1.0 ± 0.3 $_{b, D}$	1.6 ± 0.1 $_{b, D}$
Plastic (Tote)	2.5 ± 0.4 $_{a, A}$	2.9 ± 0.2 $_{b, A}$	3.3 ± 0.4 $_{b, A}$	2.8 ± 0.3 $_{b, A}$	3.2 ± 0.4 $_{b, A}$
Concrete	2.3 ± 0.2 $_{a, AB}$	2.9 ± 0.1 $_{b, AB}$	3.0 ± 0.2 $_{b, AB}$	2.6 ± 0.4 $_{b, AB}$	2.8 ± 0.2 $_{b, AB}$
Rubber (Belt)	2.0 ± 0.2 $_{a, AB}$	2.8 ± 0.1 $_{b, AB}$	2.9 ± 0.1 $_{b, AB}$	2.5 ± 0.4 $_{b, AB}$	2.8 ± 0.2 $_{b, AB}$
Rubber (Tire)	1.6 ± 0.4 $_{a, C}$	1.9 ± 0.3 $_{b, C}$	1.8 ± 0.2 $_{b, C}$	1.8 ± 0.2 $_{b, C}$	1.7 ± 0.4 $_{b, C}$

[a]Each mean is based on $n = 3$ replications.

[b]Means among the treatments across concentrations followed by different letters in lower case are significantly different ($P<0.05$, Tukey's test).

[c]Within each treatment concentration, means among contact surfaces followed by different letters in upper case are significantly different ($P<0.05$, Tukey's test).

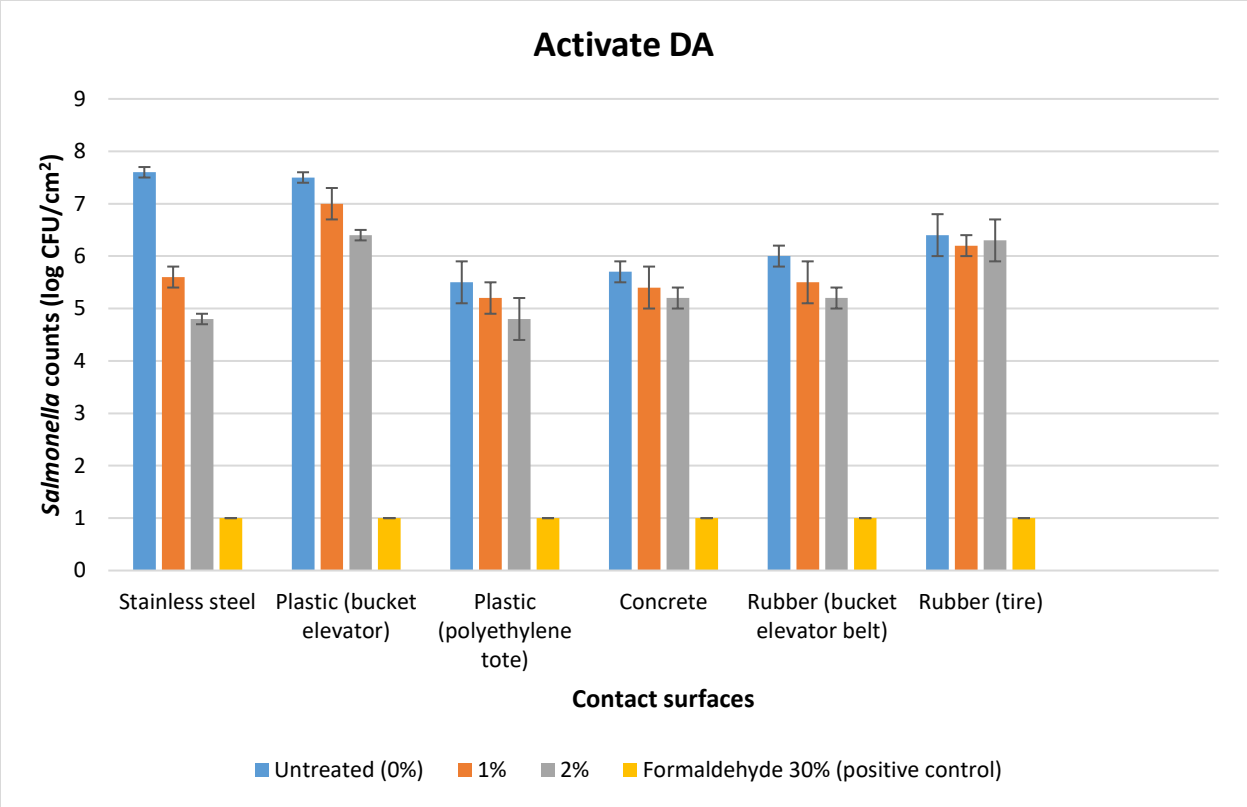

Fig. 5.1. Mean logarithmic counts (log CFU/cm^2) of *Salmonella* on food contact surfaces treated with organic acid mixture Activate DA at 1% and 2% concentrations in comparison with untreated (0% organic acid) and positive control (30% formaldehyde). Limit of detection is 1 log CFU/mL for this study.

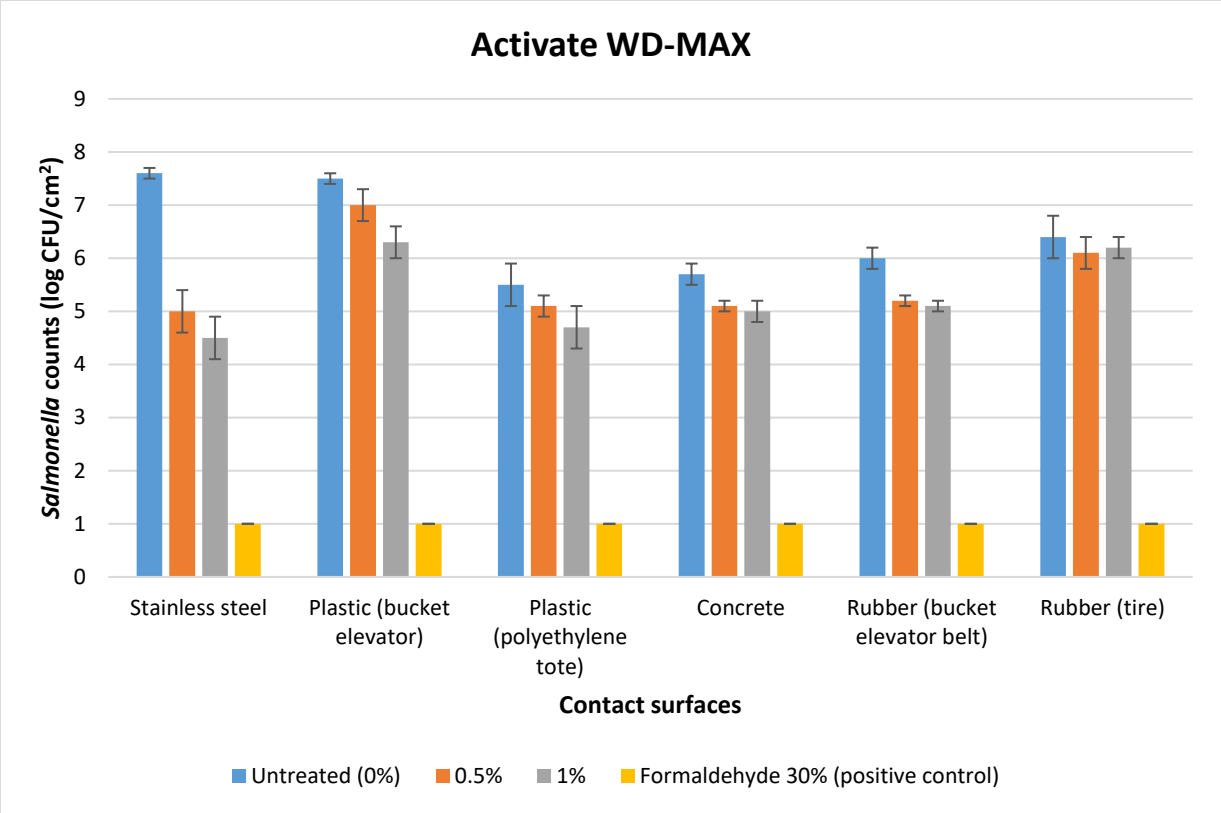

Fig. 5.2. Mean logarithmic counts (log CFU/cm^2) of *Salmonella* in food contact surfaces treated with organic acid mixture Activate WD-MAX at 0.5% and 1% concentrations in comparison with untreated (0% organic acid) and positive control (30% formaldehyde). Limit of detection is 1 log CFU/mL for this study.