AGRICULTURE, FOOD & ANALYTICAL BACTERIOLOGY

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Agriculture, Food and Analytical Bacteriology

The Efficacy of a Commercial Antimicrobial for Inhibiting *Salmonella* in Pet Food

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ABSTRACT

A commercially available antimicrobial consisting of a proprietary mixture of 5-25% (wt/vol) nonanoic acid, 1-25% (wt/vol) butyric acid, 1-50% (wt/vol) trans-2-hexenal and water was tested for efficacy against Gram-negative and Gram-positive bacteria, some isolates of *Salmonella* spp *in vitro* and activity against *Salmonella* in pet food. The *in vitro* efficacy of the antimicrobial was found to be generally effective against both Gram-positive and Gram-negative bacteria. Minimal inhibitory concentrations (MICs) were determined for isolates of *Salmonella* serotypes. Isolates of Heidelberg, Montevideo and Enteritidis had MICs of 1.5 µl/ml while the other five tested isolates had MICs of 2.0 µl/ml. The effectiveness of the antimicrobial in ground pet food artificially contaminated with a high level of *Salmonella* was assessed at 0, 1.0, 1.5, or 2.0 ml/kg of feed. Contaminated feed was sampled on days 0, 1, 4, 7 and 14 after treatment. All levels of antimicrobial resulted in nearly a 1.0 log CFU/g reduction of *Salmonella* numbers at time of treatment, and *Salmonella* levels were 2.0 log CFU/g lower at day 14 as compared to the untreated control. This antimicrobial would be useful in extending the shelf life of dried pet foods as well as limiting survival and growth of *Salmonella*.

Keywords: *Salmonella*; pet food; organic acids; butyric acid; nonanoic acid; trans-2-hexenal; antimicrobial; food safety; foodborne illness; companion animals

Agric. Food Anal. Bacteriol. 5: 65-72, 2015

INTRODUCTION

Companion animals have become an increasing aspect of the family unit in most societies. In the US alone there are an estimated 37% of households with at least one dog and 30% of households with a cat (AVMA, 2014). Most of these households feed their pets dry food for at least part of the diet (Buchanan *et al.*, 2011). Many of these dry pet foods contain ingredients of animal origin, and thus are at risk for contamination with *Salmonella* spp. Dry pet foods are made using extrusion manufacturing in which the combined ingredients are heated and formed into the final product of various shapes and sizes. The extrusion process takes place at very high tempera-

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tures which acts as a kill step for pathogens. However, high temperatures also destroy some of the nutrients present in the food, so flavor enhancers and fat, both of animal origin, are then sprayed on after extrusion. However there is no additional kill step for pathogens after this process (Thompson, 2008).

White *et al*. (2003) sampled randomly collected dog treats derived from pig ears and other animal parts in the United States and cultured them for the presence of *Salmonella*. Forty-one percent of the samples were found to be positive for *Salmonella* and 24 different serotypes were isolated from the positive samples. They isolated *S*. Infantis with PFGE patterns indistinguishable from the strains responsible for the 1999 Canadian outbreak from several products. Li *et al*. (2012) reported on the prevalence of *Salmonella* spp. in animal feeds. They isolated *Salmonella* from 6.1% of pet foods and treats, and from 7.1% of supplement-type pet products. More recently, Nemser *et al*. (2014) found only 1 of 670 dry pet foods or treats were positive for *Salmonella* spp. Nevertheless, *Salmonella* infections have been found both in pets and in humans, and were determined to be linked to contaminated pet foods and treats (Clark *et al*. 2001; CDC 2005; Behravesh *et al*. 2010; Imanishi *et al*. 2014).

In 1999 in Canada, an outbreak of *Salmonella* serotype Infantis infections in humans was found to be associated with pet treats for dogs produced from processed pig ears. Phage typing and pulsed-field gel electrophoresis (PFGE) determined that *Salmonella enterica* serotype Infantis isolated from pig ear pet treats as well as isolates from humans exposed to the pig ears were the same (Clark *et al*., 2001). Schotte *et al*. (2007) reported on a large outbreak of canine salmonellosis in German military watch dogs. The outbreak was recognized by a monitoring program and was found to be due to 2 serotypes of *Salmonella*, Montevideo and Give. Dogs in 4 kennels were exposed and 63.8% of the dogs had positive fecal samples, although only 9 dogs exhibited clinical disease. Two commercial dehydrated dog foods were implicated by risk analysis as the suspected infectious sources and *S*. Montevideo and *S*. Give with similar plasmid profiles and PFGE-restriction patterns were isolated from the suspected foods and fecal samples. In 2012 in the United States a routine sample collected of dry dog food was found to be positive for *S*. Infantis (Imanishi *et al*., 2014). The Centers for Disease Control and Prevention was able to link the genetic fingerprint of this isolate with humans with infections caused by *S*. Infantis. The subsequent outbreak investigation identified 53 ill humans infected with the outbreak strain in 21 states and 2 provinces in Canada. Traceback investigations identified one production plant as the source of the contaminated food, and the outbreak strain was isolated from unopened bags of dry dog food and fecal specimens from dogs that had eaten the food and lived with ill people.

These outbreaks confirm that large outbreaks of salmonellosis occur after feeding contaminated dry pet foods and pet treats. This also puts pet owners and vulnerable members of their households at risk as they often live in close contact with their animals. These highly publicized salmonellosis outbreaks and recalls of dry pet foods due to contamination with *S. enterica* have caused a major review of microbiological control programs, and have reinforced the idea that food safety should extend beyond traditional factory quality management processes. As in food for human consumption, ensuring the microbiological integrity of pet foods must cover the entire production pipeline ('farm-to-fork approach'). The study reported in this paper was developed to determine whether a commercial antimicrobial would restrict the survival and growth of *Salmonella* in dry dog food. The antimicrobial contains butyric acid, nonanoic acid and trans-2-hexenal.

MATERIALS AND METHODS

Determination of antimicrobial spectrum

Lyophilized cultures of test organisms (*Salmonella* Typhimurium, *Escherichia coli*, *Staphylococcus aureus*, *Clostridium perfringens*, *Lactobacillus plantarum*, *Streptococcus agalactiae* and *Campylobacter jejuni*)

were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Cultures were resuscitated according to ATCC recommended methods and transferred to Standard Methods Agar (SMA; BD Diagnostics, Franklin Lakes, NJ). Agar plates were incubated for 24 hours at 35°C.

After incubation, bacterial colonies were transferred to individual test tubes containing 10 mL of Trypticase Soy Broth (TSB; BD Diagnostics). Test tubes were incubated at 35°C for 18 to 24 hours. The level of bacteria in the broth culture was determined by serial dilution and plating on SMA. Cultures were diluted to a final concentration of 10^5 cfu ml^{-1} with Butterfield's phosphate buffer.

One mL of CO-60 surfactant and 1 mL of the antimicrobial (Preserv-8®; Anitox Corp., Lawrenceville, GA) were mixed together (the surfactant was used to allow the antimicrobial to be soluble in water for test purposes). A 0.2 ml aliquot of the mixture was added to 9.8 mL of sterile, deionized water to prepare a 1% stock solution (10 ml kg^{-1}). The stock solution was diluted with sterile deionized water to the equivalent of 5, 1, 0.5, 0.1 and 0.05 ml kg^{-1}.

A 100 µL aliquot of the 10^5 cfu ml^{-1} inoculum was added to each of the dilution tubes containing the different concentrations of antimicrobial. Tubes were vortexed for 30 seconds every hour for four hours. A 1 mL aliquot was removed from each tube at 24 hours and serially diluted in Butterfield's phosphate buffer. Dilutions were plated on selective agars as recommended for each type of bacteria. Plates were incubated at 35°C for 48 hours prior to enumeration. *Clostridium*, *Lactobacillus* and *Campylobacter* plates were incubated under anaerobic conditions.

Determination of minimal inhibitory concentration

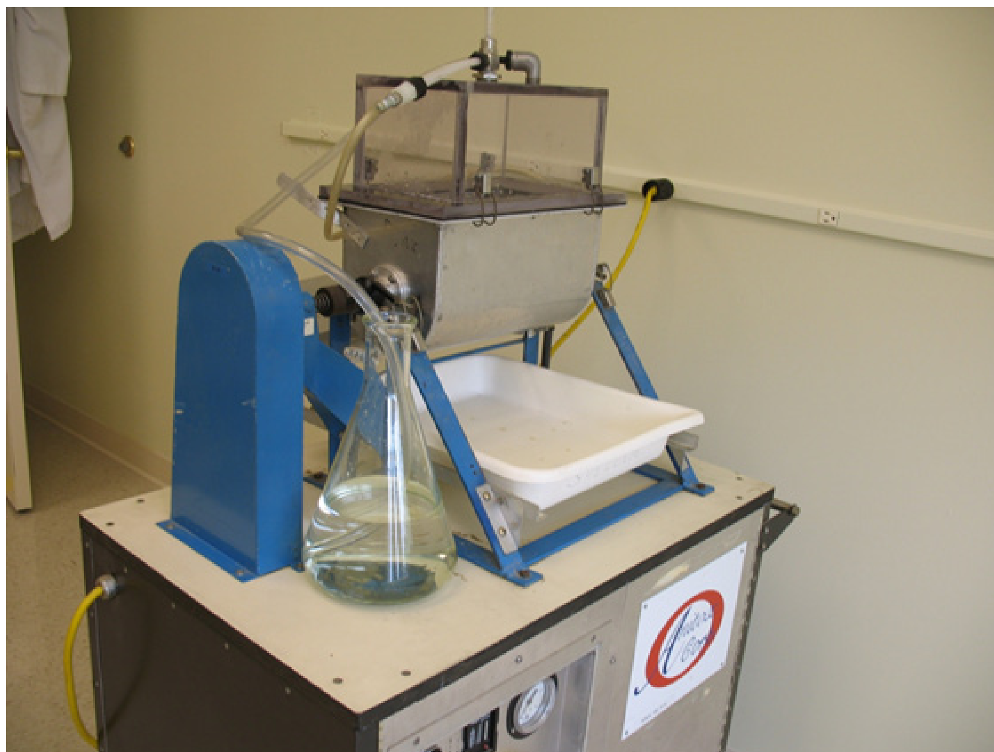
Isolates of 8 serovars of *Salmonella* were tested (Heidelberg, Montevideo, Enteritidis, Typhimurium, Worthington, Kentucky, Senftenberg and Infantis); all isolates were obtained from the culture collection of the Center for Food Safety of the University of Arkansas. Overnight cultures were prepared by inoculating 10 ml of sterile LB broth (EMD Millipore, Billerica, MA) with a single isolate of a serotype of *Salmonella* and incubating at 37°C for 18 to 24 h. Minimal inhibitory concentration (MIC) levels were determined in 96-well clear microtiter plates (NUNC, Rochester, N.Y., U.S.A.) with lids. A stock solution of the antimicrobial was prepared at 1%. Prepared sterile LB broth was aseptically pipetted (100 µl) into all wells of the microtiter plate. A 100 µl aliquot of the antimicrobial was pipetted into the first row of wells and serial 2-fold dilutions were performed to the end point of 0.25% of the antimicrobial, and 100 µl of excess solution was discarded from the last row to keep well volumes equal. One row was used as a positive control and contained 5 µl ml^{-1} of butyric acid; another row of wells was used as a negative control and contained bacteria and LB only. A 100 µL aliquot (containing approximately 10^5 cfu) of a single *Salmonella* culture was pipetted into each well. Microtiter plates were incubated statically at 37°C for 18 hours and optical density (OD) was read at 600 nm. The MIC was defined as the first well that had an OD no greater than the wells containing butyric acid. The experiment was repeated in triplicate.

Efficacy of antimicrobial in animal feed

A culture of each serovar of *Salmonella* was prepared by individually inoculating into 10 ml of sterile TSB with a single serovar and incubating in a shaking incubator at 37°C for 24 hours. One ml aliquots from each of the 8 cultures were mixed to form a cocktail. Cell density of the inoculum was adjusted to approximately 10^8 cfu ml^{-1}.

Meat and bone meal (MBM) was used as the carrier matrix to inoculate the feed. The autoclaved MBM was weighed out into 20 g aliquots and each aliquot was mixed with 90 ml of 0.01% peptone water and autoclaved. Four of the five samples were inoculated with the cocktail and shaken well. All samples were subsequently centrifuged (Beckman JR-21, Beckman Coulter, Indianapolis, IN) for 15 minutes at 27,000 x g, the excess peptone was poured off and the MBM was placed into deep petri dishes, covered with a sterile filter paper, and allowed to dry at ambient temperature for 48 hours in a biosafety cabinet.

Figure 1. Lab scale mixer used to mix antimicrobial with feed



The inoculated MBM was scraped out from the deep plates and placed in a stomacher bag and stomached to a powder. An aliquot of 10 g of the inoculum was placed with 990 g of ground dog food (from a commercial source) in a lab scale mixer (Figure 1) and mixed for 2 minutes. The antimicrobial was added to the inoculated feed using a nebulizer fitted into the mixer and at a positive air pressure of 8 PSI. The antimicrobial was injected through a septum with a 19-gauge needle. The mixer was set to a speed of 60 rpm and allowed to mix for 2 minutes. Levels of antimicrobial were equivalent to 0, 5.0, 7.5 and 10 m kg^{-1} of feed. Each group was sampled on days 0 (immediately after treatment), 1, 4, 7 and 14 after treatment.

For each sample of inoculated ground dog food mixed with antimicrobial, 1 g was placed in 9 mL of sterile 0.1 % peptone water (initial 1:10 dilution) and further diluted to the appropriate end point by serial dilution. An aliquot of 0.1 ml of each dilution was dispensed onto duplicate xylose lysine desoxycholate (XLD; BD Diagnostics) agar plates and spread with a sterile spreader. Uninoculated MBM (UMBM) was used as a negative *Salmonella* control, which was cultured as described for samples. Plates were incubated at 37°C for 24 hours and then enumerated for the amount of *Salmonella* remaining. The entire experiment was replicated three times.

RESULTS

Antimicrobial spectrum

The antimicrobial was observed to have efficacy against both Gram-positive and Gram-negative bacteria (Table 1). The degree of efficacy was similar to that obtained with formaldehyde and formic acid under similar test conditions (Carrique-Mas *et al.* 2006). A 0.05% dilution (0.5 ml kg^{-1}) of the antimicrobial gave 100% reduction of *S.* Typhimurium, *E. coli*, *S. aureus*, *S. agalactiae* and *C. jejuni* after 24 hours of exposure. *C. perfringens* and *L. plantarum* were observed to be more resistant than the other organisms, with *C. perfringens* reduced 100% at 0.1% (1 ml kg^{-1}) and *L. plantarum* reduced 100% at 0.5% (5 ml kg^{-1}).

Table 1. Results of efficacy testing of antimicrobial on various bacteria regularly found in pet food and animal feed. Initial inoculum was 5.0 log cfu/mL of bacterial culture. Exposure time was 24 hours

Treatment Level	Percent reduction compared to the control						
	S. Typhimurium	*E. coli*	*S. aureus*	*Cl. perfringens*	*L. plantarum*	*S. agalactiae*	*C. jejuni*
0.005%	36	7	43	0	0.0	92.3	6.1
0.01%	59	29	39	4	0.0	98.3	59.2
0.05%	100	100	100	85	75	100	100
0.1%	100	100	100	100	99	100	100
0.5%	100	100	100	100	100	100	100

Minimal inhibitory concentration

Minimal inhibitory concentrations of *Salmonella* serotypes varied between 1.5 µl ml^{-1} to 2.0 µl ml^{-1}, which is equivalent to 1.5 ml kg^{-1} and 2.0 ml kg^{-1} of feed respectively.

Efficacy in pet food

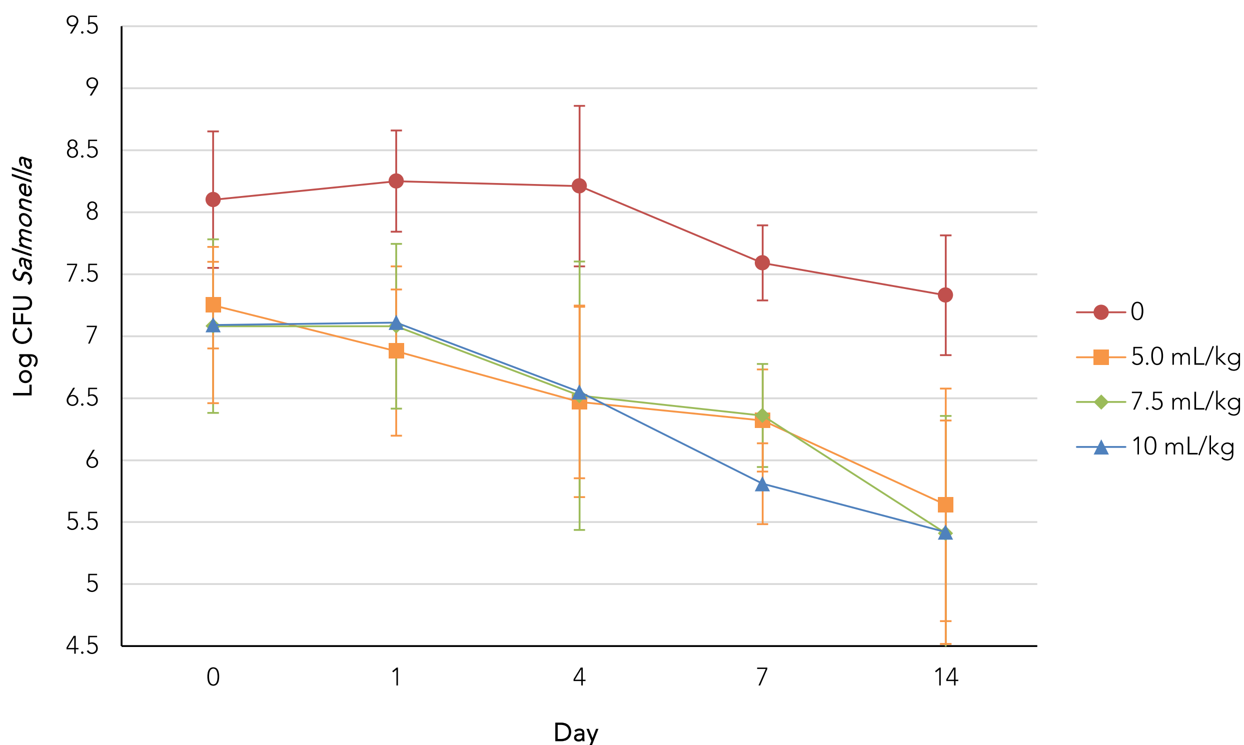
The antimicrobial inhibited *Salmonella* survival in the feed at all levels of application (Fig 2). Numbers of *Salmonella* in the untreated control increased from 8.1 log cfu g^{-1} at time 0 to 8.2 log cfu g^{-1} at 4 days. Numbers of *Salmonella* in the untreated control decreased from day 4 to day 14 with the final number being 7.3 log cfu g^{-1}. At time 0 all levels of treatment had a lower count by almost 1 log cfu g^{-1} and within 24 hours all levels of antimicrobial were a full 1 log cfu g^{-1} lower than the untreated control. At the end of 14 days all levels were close to 2.0 log cfu g^{-1} lower than the untreated feed. The UMBM was negative for *Salmonella* growth.

DISCUSSION

Organic acids are often used as preservatives of human foods (Brul and Coote, 1999) and have also been used in poultry feed to control mold and bacteria (Paster *et al*., 1987). Treatment of poultry feed with organic acids has been shown to have the potential to reduce infection levels of *Salmonella* (Khan and Katamay, 1969; Matlho *et al*., 1997). Any chemical used to control *Salmonella* in feeds must also either be metabolized by the animal or excreted without absorption (Carrique-Mas *et al*., 2007). Hume *et al*. (1993) found that organic acids used to treat poultry feed were rapidly metabolized by the birds.

Researchers have suggested that small chain fatty acids exhibit antimicrobial activity in the undissociated form because they are lipid permeable in this form and can cross the microbial cell wall and dissociate in the more alkaline interior of the microorganism making the cytoplasm unstable for survival. (Paster, 1979; Van Immerseel *et al*., 2006). Butyric acid when used alone was been found to inhibit *Sal-*

Figure 2. Efficacy of antimicrobial against a cocktail of *Salmonella* inoculated into pet food. Antimicrobial was added at 0, 5.0 ml/kg, 7.5 ml/kg or 10 ml/kg of pet food. Error bars represent standard deviation from the mean.



monella (Khan and Katamay, 1969). Khan and Khatamay (1969) found that butyric acid completely inhibited the growth of *Salmonella* in media, and when it was used to treat meat and bone meal artificially inoculated with *Salmonella* no viable organisms were recovered even after a week. Nonanoic acid (also known as pelargonic acid) is a naturally occurring fatty acid with a faint odor compared to butyric acid and is almost insoluble in water (EPA, 2004). Nonanoic acid is found in a variety of fruits as well as in dairy products, and is on the FDA generally recognized as safe (GRAS) list as a synthetic food flavoring agent, as an adjuvant, production aid and sanitizer to be used on food contact surfaces. Very few have studied the effects of nonanoic acid as an antimicrobial, but Khan and Khatamay (1969) found essentially no activity against *Salmonella* artificially inoculated into meat and bone meal.

Another volatile compound contained in the studied antimicrobial is trans-2-hexenal, which is present in many edible plants such as apples, pears, grapes, strawberries, kiwi, and tomatoes and has been an effective antimicrobial against Helicobacter pylori and *S.* Cholerasuis (Kubo *et al.*, 1999; 2001). Kim and Shin (2004) found that trans-2-hexenal (247 mg/L) was effective against *Bacillus cereus*, *S.* Typhimurium, *Vibrio parahemolyticus*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Escherichia coli* 0157:H7. Nakamura and Hatanaka (2002) demonstrated that trans-2-hexenal was effective in controlling *S.* Typhimurium at a level of 3 - 30 ug ml^{-1}. The suggested mode of action of trans-2 hexenal is the destruction of electron transport systems and the perturbation of membrane permeability (Gardini *et al.*, 2001).

Previous research has shown that the reduction of *Salmonella* in feed by treatment with organic acids may require up to a week of contact to achieve results (Iba and Berchieri, 1995). Our results suggest a

reduction of a high level of contamination with *Salmonella* within 24 hours of application by this combination of organic acids with trans-2-hexenal. Additionally, the reduction was much greater after 4 days of contact as compared to the control, where *Salmonella* growth actually increased. Wales *et al*. (2013) studied various feed treatment formulations containing organic acids and found reductions in *Salmonella* of around 1 log unit after 7 days. They also found that those formulations that ultimately had greater reductions also reduced *Salmonella* numbers much sooner, often within 24 hours of incorporation.

The tested antimicrobial was effective in feed at all levels tested regardless of MIC determined *in vitro*. All components are generally recognized as safe (GRAS) in the US, and thus are approved for use in animal feeds. This antimicrobial is a promising new treatment to reduce *Salmonella* carriage in pet foods.

ACKNOWLEDGEMENTS

This research was funded by a grant from Anitox Corp., Lawrenceville, GA.

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