Mitigation of *Salmonella* on Pet Food Kibbles by Using Liquid and Powdered 3-Hydroxy-3-Methylbutyric Acid

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ABSTRACT

In recent years, several pet food recalls have been attributed to *Salmonella* contamination. In addition to the negative impacts on animal health, *Salmonella*-contaminated pet foods have been linked to infection in humans. With that in mind, the U.S. Food and Drug Administration has set forth a zero-tolerance policy for *Salmonella* in pet foods. Typically, pet foods are extruded or processed at high temperatures that are sufficient to reduce pathogenic bacteria. However, the possibility for postextrusion contamination still exists. One potential method to reduce the risk of postextrusion contamination of pet foods with *Salmonella* is through the addition of a chemical additive coating. The objective of this research was to evaluate the ability of β-hydroxy-β-methylbutyrate (HMB), in either free acid (HMBFA) or calcium salt (CaHMB) form, to reduce postextrusion contamination of dry extruded dog kibble with *Salmonella*. Three trials were conducted with HMBFA and CaHMB coated onto the kibbles at levels of 0, 0.1, 0.3, 0.5, 0.9, and 1.5% (w/w). The coated kibbles were then inoculated with *Salmonella enterica* subsp. enterica Enteritidis (ATCC 13076), with enumeration done on days 0, 1, 2, 7, and 14 postinoculation. Subsamples on each day were serially diluted, spread plated to xylose lysine deoxycholate agar, and incubated at 37°C for 24 h. *Salmonella* colonies were then counted and log CFU per gram was calculated. The 1.5% HMBFA reduced counts by 4.9 ± 0.2 log units on day 1, whereas the positive control only decreased 2.2 ± 0.1 log units (*P* < 0.0001). The 1.5% CaHMB level decreased counts by 7.1 ± 0.04 log units by day 7 compared with the control decrease of 2.1 ± 0.1 log units (*P* < 0.0001). All HMBFA and CaHMB treatments resulted in the elimination of detectable *Salmonella* counts by day 14 (*P* < 0.0001 versus controls). In conclusion, HMB coating was effective at reducing *Salmonella* artificially inoculated to dog kibbles in a model of postextrusion contamination.

Key words: β-Hydroxy-β-methylbutyrate; Calcium salt form of β-hydroxy-β-methylbutyrate; Mitigation; Pet food; *Salmonella*

The Centers for Disease Control and Prevention estimates that *Salmonella* causes ~1.2 million human foodborne illnesses and 450 deaths each year in the United States (9). Although *Salmonella* infections are traditionally linked to the consumption of contaminated meat or poultry, more recently several outbreaks have been linked to vegetable products, live animals, and to dry pet foods and treats (2, 5–8, 13). To help reduce the risk to humans, the recently implemented Food Safety Modernization Act has shifted the focus of food and feed safety from reactive to preventative (18). These new regulations have put increased scrutiny on animal foods, including pet foods. With the introduction of new Food Safety Modernization Act regulations, a zero-tolerance policy for *Salmonella* in pet foods has been established (19).

Although low moisture foods, including pet foods, are generally considered shelf stable and safe to consume without further processing, they commonly contain ingredients that may be at high risk for *Salmonella* contamination (13, 15). Because of this risk, many pet food manufacturers rely on rigorous process controls and current good manufacturing practices to reduce this risk. The heat and pressure introduced during thermal processing via conditioning and extrusion can substantially reduce *Salmonella* (3, 4, 13). However, this type of processing only offers a point-in-time mitigation of *Salmonella*. Thermally processed kibbles are still at risk for postextrusion cross-contamination with *Salmonella*. The potential for postextrusion contamination can occur during kibble coating, cooling, packaging, and storage (10, 11).

Even when *Salmonella* is present, storage conditions at lower moisture and at room temperature do result in a decline in *Salmonella* population with time; however, significant levels of viable organisms can still be present up to 19 months after inoculation of a commercial dry dog food product (14). The ability of postextrusion contamination to persist until product use dictates that a means of reducing the potential for *Salmonella* survival postprocessing must be used to ensure that end users will not come into contact with viable organisms. Contaminated pet food has resulted in cross-species salmonellosis (2), and one such
outbreak from 2006 to 2008 in the United States resulted in 79 known cases of human illness from contaminated dry dog and cat food (2, 6, 7).

In contrast to physical processing, chemical additives have the potential for more long-term mitigation potential against Salmonella and other pathogens. In the animal feed industry, chemical additives are often derived from blends of organic acids or commercial formaldehyde (10–12, 20). More recently, Cochrane et al. (10) saw a reduction of Salmonella contamination in protein meal feed ingredients with the inclusion of a medium chain fatty acid blend, organic acid blend, or an essential oil blend. In addition, coating of pet food kibbles with the dry acidulant sodium bisulfate was found to effectively reduce the survival of Salmonella artificially inoculated to the treated kibbles (10). 3-Hydroxy-3-methylbutyrate (HMB) is a small organic acid available in both free acid liquid (HMBA) and powdered calcium salt (CaHMB) forms. Both forms of HMB are chemically synthesized and both are self-affirmed generally recognized as safe for humans. Much of the current body of research on HMB focuses on its function as a metabolite of the essential amino acid leucine (16, 17, 21); however, similar to other organic acids used, HMB may also have antimicrobial properties when used in animal foods and feeds.

The objectives of this study were first to evaluate the potential of liquid HMBA and powdered CaHMB to mitigate Salmonella in HMB-coated kibbles and second to determine the minimum effective inclusion level for Salmonella reduction in coated kibbles.

MATERIALS AND METHODS

Three studies were conducted to evaluate the efficacy of HMBA and CaHMB to mitigate Salmonella in kibble. The first two studies used HMBA and were conducted at Kansas State University (Manhattan). The third study used CaHMB and was conducted at the Eurofins Microbiology Laboratories (Lancaster, PA). The same protocols were used by both laboratory facilities.

Kibble coating. All kibbles were coated with HMBA or CaHMB at Kansas State University. The HMBA and CaHMB were provided by Metabolic Technologies, Inc. (Ames, IA). Study 1 was conducted as a preliminary evaluation to determine the potential for HMBA coated on kibbles to mitigate Salmonella. For study 1, kibbles previously manufactured at Kansas State University from 100% sorghum flour were coated with liquid HMBA by using a bench-top paddle mixer (Cabela’s, Sidney, NE). The HMBA was applied to the kibbles at inclusion levels of 0, 0.9, and 1.5% (w/w; calculated before application to kibble). For application, the designated weight of HMBA was diluted with sterile distilled water to a total volume of 20 mL for the 0.9 and 1.5% treatments before application to the kibbles. For the 0% treatment (positive control), 20 mL in total of sterile distilled water was coated on the kibbles with continuous mixing. All liquids were applied directly to the kibbles during mixing via a spray applicator attached to the lid of the mixer. Upon completion of the liquid application, kibbles were mixed for an additional 5 min to ensure an even distribution of the HMBA. For liquid HMBA application, no residual liquid in the mixer remained after kibble coating. Coated kibbles were then allowed to dry on trays for at least 2 h, placed in plastic containers, and stored at room temperature during the three studies. During the 14-day storage, temperatures were ~20 to 25°C and relative humidity ranged from ~20 to 35%. Incubation of Salmonella onto coated kibbles was done the day after kibble coating.

For study 2, kibbles (all life stages diet) were collected from a commercial pet food manufacturer before the fat-coating step. Again, the kibbles were coated with liquid HMBA as described in study 1, but at inclusion levels of 0, 0.1, 0.3, and 0.5% (w/w), followed by drying for 2 h and storing in plastic containers at room temperature. Incubation of coated kibbles with Salmonella was again performed on the next day.

For study 3, the commercially manufactured kibbles were again used and coated with CaHMB at inclusion levels of 0, 0.1, 0.3, 0.5, 0.9, and 1.5% (w/w; calculated before application to the kibble). In brief, during mixing of the kibbles, the CaHMB was sprinkled over the kibbles and mixed until all the CaHMB powder had adhered to the kibbles. Again, no residual CaHMB remained in the mixer after application to kibble. After CaHMB coating, the coated kibbles were stored in plastic containers at room temperature. The kibbles were sent to Eurofins, and day 0 was the day the kibbles were inoculated with Salmonella in the laboratory.

All kibbles were determined to be Salmonella negative before being used in the study by using the methods adapted from the U.S. Food and Drug Administration’s Bacteriological Analytical Manual, Chapter 5 (1). For all studies, the same method was used for preparation of the Salmonella inoculum, kibble inoculation, and enumeration over time. All Salmonella inoculations to the coated kibbles were done in triplicate. For the preparation of Salmonella inoculum, a stock culture of Salmonella enterica subsp. enterica Enteritidis (ATCC 13076) maintained at ~80°C was transferred to Trypticase soy broth (TSB; BD, Franklin Lakes, NJ) and incubated at 37°C for 48 h. To prepare the culture for inoculation onto the coated kibbles, the culture was centrifuged at room temperature at 5,452 × g for 10 min to pellet the cells, and all but 3 mL of the supernatant was removed. The pelleted cells were resuspended in the remaining 3 mL of TSB in studies 1 and 2 and in 10 mL of TSB in study 3. The inoculum was then transferred to a hand-pump spray applicator for inoculation onto the kibbles. A separate inoculum was prepared for each HMBA and CaHMB inclusion level and each of the three replicates per treatment level. Incubation of the coated kibbles (150 g) was done by spraying the entirety of the prepared inoculum directly onto the kibbles. A negative control was also included for each study and consisted of sterile TSB inoculated onto uncoated kibbles. After inoculation, the kibbles were shaken to thoroughly distribute the inoculum. Inoculated kibbles were allowed to equilibrate at room temperature for 2 h before subsampling for the day 0 enumerations. The remaining coated and inoculated kibbles were stored (see above) for enumeration on days 1, 2, 7, and 14 postinoculation. For all studies, a subsample was collected from each of the three replicates at each treatment level and evaluated for Salmonella.

Enumeration of Salmonella was done by removing a 25-g subsample of the coated and inoculated kibbles and homogenizing with buffered peptone water (BD) for 15 s in a stomacher (Stomacher 400, Seward, Islandia, NY) for studies 1 and 2. For study 3, the 25-g subsamples were shaken vigorously instead of using a stomacher. The homogenate was then serially diluted using buffered peptone water and spread plated onto xylose lysine deoxycholate agar (BD) in duplicate and incubated at 37°C for 24 h. After incubation, plates were enumerated by counting black colonies, typical for Salmonella. The number of observed colonies was averaged across both plates and was multiplied by the dilution.
factor to determine the total count in CFU per gram of kibble. Representative isolates from day 0 were confirmed as Salmonella serovar Enteritidis through serotyping to ensure the recovered counts represented the inoculated culture. For studies 1 and 2, collected isolates were sent for serotyping to the National Veterinary Services Laboratory (Ames, IA). For study 3, the Eurofins Microbiology Laboratories confirmed the isolates as Salmonella serovar Enteritidis.

To further evaluate the Salmonella-mitigating properties of HMBFA and CaHMB, all treatment samples with no detectable Salmonella were enriched overnight to allow damaged Salmonella cells to be recovered. In brief, after initial plating of the samples, the first dilution (25 g of sample plus 225 mL of buffered peptone water) was placed in the incubator for enrichment for 24 h. Enriched samples were then spread plated to xylose lysine deoxycholate agar, incubated at 37°C for 24 h, and growth of black colonies typical for Salmonella were noted (presence–absence result, not quantitative).

**Statistical analysis.** The results were calculated as CFU per gram of kibble and converted to log values for statistical analysis. The GLIMMIX procedure in SAS 9.4 (SAS Institute Inc., Cary, NC) was used with the treatment concentration as the fixed effect and enumeration day serving as a repeated measure. All data are

**FIGURE 1.** Salmonella levels (log CFU per gram) in the coated kibbles on each sample enumeration date. Each data point is the mean of three replicates, with the standard error of the mean indicated with error bars. (A) Study 1 results with the β-hydroxy-β-methylbutyric acid (HMBFA) coating at either 0 (positive control) (■), 0.9% (●), or 1.5% (+). The minimum level of detection was 100 CFU/g on enumeration day 1 and 10 CFU/g on days 2, 7, and 14. Main effects were treatment (Trt) P < 0.0001, enumeration day (Day) P < 0.0001, and Trt × Day P < 0.0001. (B) Study 2 results with the HMBFA coating at either 0% (positive control) (■), 0.1% (▲), 0.3% (×), or 0.5% (○). The minimum level of detection for studies 2 and 3 was 10 CFU/g on all enumeration days. Main effects were Trt P < 0.0001, Day P < 0.0001, and Trt × Day P < 0.008. (C) Study 3 results with the calcium β-hydroxy-β-methylbutyrate (CaHMB) coating at either 0% (positive control) (■), 0.1% (▲), 0.3% (×), 0.5% (○), 0.9% (●), or 1.5% (+). Main effects were Trt P < 0.0001, Day P < 0.0001, and Trt × Day P < 0.0001.
presented as the arithmetic mean of the three replications ± standard error of the mean. Treatment differences were considered statistically significant if \( P < 0.05 \).

**RESULTS**

In study 1, the results of the *Salmonella* enumeration for each treatment were averaged across replications on each enumeration day (Fig. 1A). Day 0 *Salmonella* counts were 6.99 ± 0.03, 5.59 ± 0.22, and 4.88 ± 0.19 log CFU/g for the 0 (positive control), 0.9, and 1.5% HMBFA treatment levels, respectively. For HMBFA treatment levels of 0.9 and 1.5%, day 1 enumerations were below the limit of detection (<100 CFU/g). Enumerations on days 2, 7, and 14 showed that both HMBFA treatments (0.9 and 1.5%) remained below the limit of detection (<10 CFU/g). By day 14 the positive control replicates averaged 3.14 ± 0.14 log CFU/g, a decrease of almost 4 log over the study period. Based on the results from enumerations on days 1, 2, 7, and 14, the enriched samples were positive on days 1 and 2, but they were negative on days 7 and 14 for *Salmonella* growth. All isolates collected from enumerations were confirmed *Salmonella* serovar Enteritidis via serotyping.

The average *Salmonella* enumeration results for study 2 are shown in Figure 1B. Day 0 *Salmonella* counts were 5.84 ± 0.05, 5.61 ± 0.19, 5.62 ± 0.06, and 5.60 ± 0.26 log CFU/g for the 0 (positive control), 0.1, 0.3, and 0.5% HMBFA treatment levels, respectively. *Salmonella* enumerations were under the limit of detection (<10 CFU/g) for the 0.5% HMBFA treatment on days 2, 7, and 14 and on day 14 across all other treatments. By day 14, the positive control replicates averaged 1.00 ± 0.51 log CFU/g, a decrease of ~5 log over the study period. All enriched subsamples from the HMBFA treatment levels below the limit of detection on enumeration days 2, 7, and 14 were positive for *Salmonella* growth. Again, all isolates collected from enumerations were confirmed *Salmonella* serovar Enteritidis via serotyping.

In study 3, CaHMB was used to coat the kibbles, and the average *Salmonella* enumeration results are shown in Figure 1C. Day 0 *Salmonella* counts were 7.25 ± 0.05, 7.19 ± 0.04, 7.17 ± 0.07, 7.09 ± 0.04, 7.08 ± 0.12, and 7.04 ± 0.13 log CFU/g for 0 (control), 0.1, 0.3, 0.5, 0.9, and 1.5% CaHMB treatment levels, respectively. Enumerations on day 7 showed the 0.5, 0.9, and 1.5% CaHMB-coated kibbles had counts below the limit of detection, and by day 14 all CaHMB-treated kibbles had enumeration counts below the limit of detection (<10 CFU/g). By day 14, the positive control replicates averaged 3.08 ± 0.18 log CFU/g, a decrease of ~4 log over the study period. All enriched subsamples, days 7 and 14 for the 0.5, 0.9, and 1.5% and day 14 for the 0.1 and 0.3% CaHMB-treated kibbles, showed no *Salmonella* growth. Again, all isolates collected from enumerations were confirmed *Salmonella* serovar Enteritidis via serotyping.

In each of the three studies, negative controls (non-inoculated) were also processed and analyzed. No *Salmonella* was detected in the negative controls during any of the studies.

**DISCUSSION**

These studies were conducted to test the effectiveness of HMB as an antimicrobial kibble coating by using a model of postprocessing contamination with *Salmonella* (10). Recently, HMB has become available in the liquid free acid form that has similar acidic characteristics as other small organic acids previously demonstrated to be effective in reducing *Salmonella* in the model used in these studies (12). The first study conducted with HMBFA at levels of 0.9 and 1.5% (w/w) coated onto the kibbles eliminated *Salmonella* inoculated onto the kibbles within 24 h. The second study was then carried out to determine a minimally acceptable coating rate with activity against *Salmonella*. It was determined that a coating level as low as 0.1% (w/w) was effective in eliminating the *Salmonella* inoculum by day 14 of the study. As dry powdered coatings are preferable in some manufacturing systems, a third study was conducted to test the efficacy of HMB in the powdered form, CaHMB. The powdered form was also very effective in attenuating the *Salmonella*, with the lower levels eliminating the *Salmonella* after 14 days, whereas the higher levels eliminated the *Salmonella* during the first week after inoculation.

In the current studies, even the lowest levels of HMBFA and CaHMB coating resulted in a 6- to 7-log decrease in *Salmonella* to undetectable levels in 14 days. Lambertini et al. (14) studied the long-term kinetics of *Salmonella* survival on kibble stored at room temperature and did not see a 4-log decrease until ~150 days. Although in our model system we did observe a 4- to 5-log decrease in *Salmonella* in our controls in the 14-day study period, this was similar to a study by Cochrane et al. (10) who used a similar model and also observed a 4-log decrease after 14 days. They also observed a 4.5-log decrease with 3% added organic acids in 14 days and elimination of the *Salmonella* at 21 and 42 days, whereas *Salmonella* persisted in the control group after the 42-day period. It seemed that for surface inoculation, the HMB acid coatings in either liquid or powder forms were more effective at a lower dosage rate; however, it is unknown what rate may be necessary in a complete feed.

This study does have limitations, the first being the assumption that postextrusion contamination will occur at the surface of the kibble where the HMB coating was applied. Although surface contamination is a likely route postextrusion, it cannot be ruled out that contamination could occur below the surface of the kibble in further handling and packaging of the kibble after coating. In addition, this study used only a surface coating application; further study is necessary to determine effective dosages for HMB when mixed in a meal type feed. It is anticipated, though, that the acidulant activity would persist when mixed in a meal type feed. However, the proximity and contact with the contaminating *Salmonella* organisms would be altered. Another area for further study is that the studies here do not perfectly replicate the activities currently practiced during kibble production. Although we have shown HMB can mitigate *Salmonella* on pet food kibble when applied directly to the surface, it is unknown whether HMB can be mixed with other common kibble coatings and still maintain
the robust anti-Salmonella activity we observed. Last, a wet Salmonella inoculum was used; whether the performance of a dry surface inoculum may behave differently is not known.

In conclusion, based on the results of the current study, HMBA levels as low as 0.1% (w/w) were effective in preventing postextrusion contamination of the kibble with Salmonella within the 14-day study period. In addition, the CaHMB also was effective in eliminating the Salmonella over the study period, albeit at a slightly slower rate than the liquid HMBA. Therefore, HMB in either form may be an effective alternative for pet food manufacturers to reduce the risk of postextrusion Salmonella contamination in dry pet food.

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REFERENCES


