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Assessing the potential for *Salmonella* growth in rehydrated dry dog food

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Abstract

Background: A substantial percentage of dog owners add water to dry dog food to increase its palatability. The recent association of *Salmonella* contamination of dry pet foods with salmonellosis cases in both dogs and their owners has generated a need to determine the ability of *Salmonella* to grow in eight commercial brands of rehydrated dry dog food.

Results: Eight brands of commercial dry dog food were rehydrated to 20, 35 and 50% added moisture, inoculated with two *S. enterica* strains (~10^5 CFU/g) and incubated for 72 h at 18 °C, 22 °C, or 28 °C. Dog food brand, moisture content, and temperature affected pathogen growth/survival patterns. Rehydration to 20% moisture did not support growth of *S. enterica*, and in general there was a 0.5–2.0 Log decline. At 35% moisture and 28 °C, 4 of 8 brands supported up to 3.4 Log(CFU/g) of growth, while *Salmonella* levels declined in three brands, and remained unchanged in one. Rehydration to 50% moisture at 28 °C supported increases of up to 4.6 Log(CFU/g) in 5 of 8 brands. Growth kinetics determinations with two of the brands that supported growth had calculated lag times, generation times, and maximum population densities of 4.4 and 2.2 h, 1.4 and 10.8 h, and 7.3 and 6.9 Log(CFU/g) when rehydrated to 35% moisture and held at 30 °C.

Conclusions: Results of this study establish that the rehydration of dry dog food with sufficient amounts of water may support the growth of *S. enterica*. Based on the most rapid observed lag times, growth of *Salmonella*, if present, in rehydrated dog food could be avoided by discarding or refrigerating uneaten portions within 2–3 h of rehydration. These data allow accidental or intentional rehydration of dry dog food to be factored into predictive microbiology models and exposure assessments.

Keywords: Pet food, Pet owners, Rehydration, Salmonellosis, Home environment, Growth kinetics

Background

Many of the forty-three million U.S. households who own dogs rely on dry pet food as their pet's primary source of nutrition (AVMA American Veterinary Medical Association 2012). Investigations of two related, multi-state outbreaks of human salmonellosis in 2006 and 2008 identified dry dog food as the source of infection (CDC U.S. Centers for Disease Control and Prevention 2008). With seventy-nine case-patients identified in 21 states of the U.S., this outbreak underscored the importance of proper handling and storage of pet foods in the home to prevent human infection (Behravesh et al. 2010). A third multi-state outbreak of salmonellosis was linked to a different manufacturer (CDC U.S. Centers for Disease Control and Prevention. Multistate outbreak of human *Salmonella* Infantis infections linked to dry dog food final update 2012; FDA U.S. Food and Drug Administration. Investigation of multistate outbreak of human infections linked to dry pet food 2012). These outbreaks, ongoing recalls of pet foods and treats, and surveys of pet foods and animal feeds have increased consumer concerns about the safety of these products (Finley et al. 2006; Adley et al. 2011; Buchanan et al. 2011; Li et al. 2012; Lambertini et al. 2016a). This, in turn, has led to a need to better understand the microbiological characteristics of dry pet food and treats.

Salmonella is noted for its ability to survive for extended periods in dry food products (Tamminga et al. 1977, Juven et al. 1984, Hiramatsu et al. 2005), including dry pet food (Lambertini et al. 2016b). During the

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development of "what-if" scenarios for an exposure assessment for *S. enterica* in dry dog food (Lambertini et al. 2016c), the purposeful or incidental wetting of the product was evaluated as a potential factor affecting the levels of *Salmonella* in the product in the home environment, and hence potential human and pet exposure. Based on a evaluation of questions asked by pet owners posted on the Internet, the instructions for moistening on dog food packages, and the veterinary literature (Laflamme et al. 2008), it appears that a substantial, though unquantified portion of dog owners moisten dry dog food and/or mix it with wet food prior to feeding. Furthermore, rehydrated pet food can remain at room temperature for substantial periods before being consumed or discarded, thereby potentially increasing levels of *S. enterica* in the home environment. However, no data were available to quantify the degree and extent of *S. enterica* growth under such circumstances.

Accordingly, the objective of the current study was to preliminarily characterize the growth of *S. enterica* in a range of dry dog foods after rehydration, and provide a means of including this factor in risk assessments.

Methods

A complete factorial design (3 × 3 × 8 × 3) with three variables – moisture level, storage temperature and brand of dog food - was used to examine *Salmonella* survival/growth in eight commercial dog food brands. Temperatures of 18, 22, and 28 °C were selected to mimic the range of temperatures that might be encountered during different seasons. Added moisture levels were set at 20, 35, and 50% added water.

Analysis of background microflora of commercial dry dog foods

A 5-lb bag of each of the eight brands of dog food was purchased at a local supermarket after careful visual examination to ensure the packages were undamaged. Upon opening of the bags in the laboratory, samples of the dog food were analyzed for *Salmonella* and aerobic plate counts using the methods described below in conjunction with the standard cultural and confirmatory techniques from the FDA Bacteriological Analytical Manual were used to screen for *Salmonella* (Andrews et al. 2016). The brands were also tested for water activity and pH (see below).

Bacterial strains used and preparation of inocula

In all tests, dry dog food was inoculated with a cocktail of two *S. enterica* strains: *Salmonella enterica* serovar Typhimurium CVM98 (animal isolate) and *Salmonella enterica* serovar Enteritidis KPL13076 (clinical isolate originally obtained from CDC). These strains were selected based on their ability to survive for extended periods in dry environments (Oni et al. 2015). An exception was one growth kinetics study at 30 °C which was conducted using *S.* Typhimurium CVM 98 and *S.* Typhimurium LT-2, due to the loss of *S.* Enteritidis KPL13076 stock culture during a power failure in our −80 °C freezer. *S.* Typhimurium LT-2 is a laboratory strain used extensively for studying *Salmonella* detection and behavior in various foods including dry foods undergoing extended dry storage. The strains were acquired from the culture collection of the Department of Nutrition and Food Science, University of Maryland. After activating individual stock cultures of both *Salmonella* strains by streaking onto Brain Heart Infusion Agar (BHIA) plates (Becton Dickinson, Sparks, MD) and incubating at 37 °C for 18 to 24 h, a single colony of each strain was selected from each plate, streaked onto separate plates of Xylose Lysine Desoxycholate Agar (XLDA) (Becton Dickinson), and incubated at 37 °C for 24 h. Single black colonies were selected from the XLDA plates and used to inoculate five 10-ml tubes of BHI broth (Becton Dickinson), which were then incubated at 37 °C for 24 h. The five 10-ml tubes were combined in a sterile 50-ml centrifuge tube (BD Falcon, Franklin Lake, NJ), and centrifuged at 3,000 × *g* for 10 min at 7 °C. Cell pellets were washed three times with 5 ml of sterile 0.1% peptone water and re-centrifuged, and the final cell pellet was re-suspended in 3 ml of sterile 0.1% peptone water. Equal volumes of each strain were combined, re-centrifuged, and re-suspended in 1 ml of sterile 0.1% peptone water to produce the two-strain cocktail with a final concentration of approximately 10^9 CFU/ml.

Preparation of samples and measurement of *Salmonella* growth/survival at specified moisture levels

The pH and water activity (a_w) of each dry dog food brand was measured upon opening each bag. pH was determined at 25 °C by weighing 1 g portions, pulverizing with a wooden mallet, hydrating with distilled water (1:2.5 g/v), and using a pH meter (Orion pH electrode 9165 BN, Orion Research, Boston, MA, USA) to take measurements. A water activity meter (Novasina IC-500, AW-LAB, Switzerland) was used to measure water activity using the manufacturer's specifications.

Three 140-g portions of each brand of dog food, one for each target moisture level, were weighed into sterile plastic bags. The appropriate amounts of sterilized distilled/deionized water were added to rehydrate the dog food samples to 20, 35, 50% added water as calculated based on the initial weight of the dog food. No attempt was made to have all the brands have the same percent moisture or water activity after rehydration. Working under a biosafety hood, 10 μl (0.01 ml) of the concentrated *Salmonella* cocktail was transferred to the water to be added to the dog food. After vortexing for 20 s,

the diluted inoculum was gradually added to the corresponding dog food sample. With each addition, the bag was gently massaged and shaken to ensure a homogeneous distribution. Portions (~10 g) of inoculated dog food were transferred to triplicate labeled plastic containers and stored for 72 h at 18, 22 and 28 °C. Three 10-g samples of each moisture level were analyzed immediately to determine the initial *Salmonella* population densities. After incubation, each 10-g sample was transferred to a Whirlpak bag (Nasco, Fort Atkinson, WI), mixed with 90 ml of sterile 0.1% PW, and stomached for 30 s. The supernatant was used to make serial dilutions, after which 50 μl aliquots of appropriate dilutions which were spiral-plated in duplicate on BHIA (total aerobic bacteria) and XLDA (*S. enterica*). This dual media plating system was used to allow estimation of the degree of injury of salmonellae recovered from the samples (Oni et al. 2015). Plates were incubated at 35 °C for 48 h, and enumerated at 24 and 48 h using an automated colony counter (Neutec Group Inc., Farmingdale, NY).

Growth kinetics of *Salmonella* in dry dog food

Two additional studies to more closely examine the growth kinetics of the *S. enterica* were carried out with dog food brands #2 and #4 at the 35% rehydration level. A rehydration level of 35% was selected as the level most likely used by consumers, based on an informal survey of pet owners in our laboratory. In the first study, the strong temperature dependency of *S.* Typhimurium CVM98/*S.* Enteritidis KPL13076 growth in brand #4 (Fig. 1b) was followed over 72 h using four temperatures: 15, 20, 25, and 30 °C. Growth was measured over 72 h by periodically taking duplicate samples and quantifying *Salmonella* population densities as described above. The second related study examined the growth kinetics of *S.* Typhimurium CVM98/*S.* Typhimurium LT-2 in brand #2 rehydrated to 35% and incubated at 30

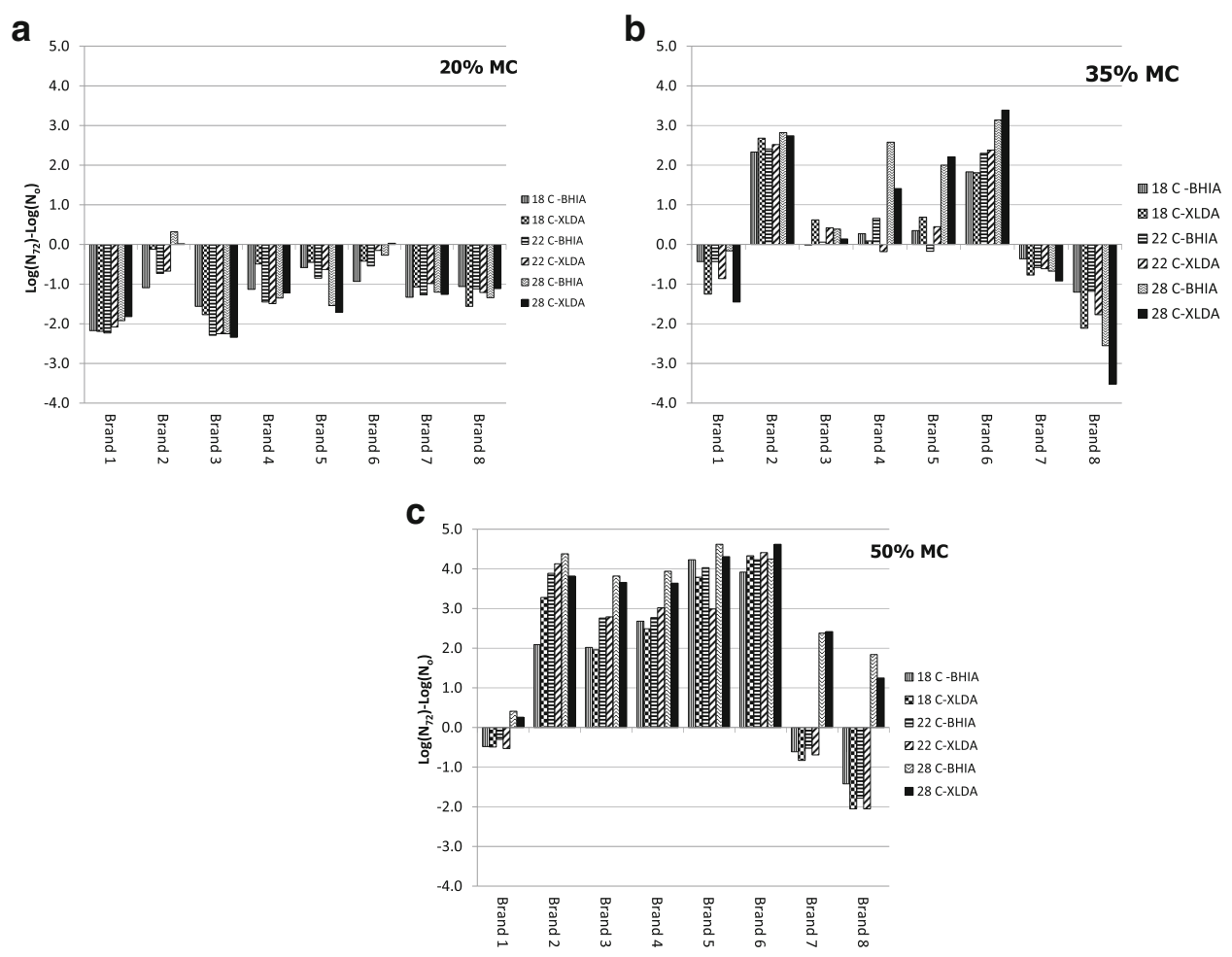

Fig. 1 Comparison of normalized growth and/or survival [Log (N_{72}) – Log (N_0)] of a cocktail of *S.* Typhimurium CVM98 and *S.* Enteritidis KPL13076 in eight brands of dry dog food at added moisture levels of (**a**) 20%, (**b**) 35%, and (**c**) 50%, and incubated at 18, 22 and 28 °C for 72 h

Table 1 Water activity and pH values of dry dog food brands

	Dog Food Brand							
	#1	#2	#3	#4	#5	#6	#7	#8
pH	5.30 ± 0.02[a]	5.52 ± 0.01	5.70 ± 0.02	5.83 ± 0.02	6.13 ± 0.02	6.12 ± 0.04	5.39 ± 0.04	5.02 ± 0.02
a_w (no added water)	0.495 ± 0.012	0.486 ± 0.010	0.401 ± 0.008	0.492 ± 0.009	0.459 ± 0.003	0.434 ± 0.009	0.661 ± 0.009	0.653 ± 0.010

[a]Values represent the mean and standard deviation of three samples

°C as described above. This brand was selected because it was one of the brands that supported substantial growth at this rehydration level. *Salmonella* counts obtained from XLDA plates were log-transformed to Log(CFU/g), and the time series fitted to the three-phase linear model (Buchanan et al. 1997) using the IPMP-2014 software (USDA/ARS, Wyndmoor, PA).

Results and Discussion

The measured pH and a_w of the eight brands of dry dog food used in this study are provided in Table 1. No *Salmonella* were detected in the uninoculated dog food (lower limit of detection ~50 CFU/g). The aerobic plate counts (BHIA plates) of the uninoculated dog food were generally below the limit of detection (~50 CFU/g), with those showing growth having counts on average of ~3.8 Log(CFU/g), and consisting largely of molds and yeast (data not shown). The measured a_w of the eight brands after addition of 20, 35, and 50% water is provided in Table 2. Visual observations of the effect of rehydration on the appearance and texture of dog food indicated that with 20% added moisture there was hardly any noticeable effect. Conversely, a moisture level of 50% resulted in the dog food having a soggy appearance and texture. Rehydration to 35% moisture yielded food pellets that were moist yet firm.

All three variables—dog food brand, moisture content, and storage temperature—influenced pathogen growth and survival patterns (Fig. 1). Clear differences were observed among the eight dog food brands. At the lowest added moisture level of 20%, a 0.5–2.0 Log decline in *S. enterica* levels was observed after the 72 h incubation (Fig. 1a). The extent of the reduction was similar at cool (18 °C), room (22 °C) or warm (28 °C) temperatures. At the 35% rehydration level, four of the eight dog food brands supported *Salmonella* increases up to 3.4 Log(CFU/g), while *Salmonella* levels declined in three brands and remained largely unchanged in brand #3 (Fig. 1b). In three of the four brands that supported growth, the extent of growth was enhanced by incubation at 28 °C. In the three brands that declined at 35% moisture, there was evidence of approximately 1 Log of injury based on the differences in BHIA and XLDA counts. Injury seemed to be enhanced at the warmer incubation temperatures. When rehydrated to 50% moisture, increases up to 4.6 Log(CFU/g) were observed in five of eight brands, with the extent of growth being enhanced by the warmer incubation temperatures (Fig. 1c). Brand #7 and brand #8, which showed substantial reductions in *Salmonella* levels at 35% moisture, supported substantial growth at 50% rehydration and 28 °C, but continued to show 1–2 Log reductions at the lower temperatures. Brand #1 remained largely unchanged over the 72-h incubation period, though to a much lesser degree it showed a response pattern similar to brands #7 and #8.

The decreases observed across all brands at the 20% rehydration level suggest a general effect. One possibility is that this level of moisture was sufficient to support increased metabolic activity but was insufficient to support growth. This hypothesis is supported by pH and a_w values after rehydration (Table 2). After 20% rehydration, the observed a_w values ranged from 0.92 to 0.97. In such a situation, the increased stress of active metabolism under the pH/a_w conditions could lead to physiological damage. However, when the moisture content was

Table 2 Water activity of dog food brands after the addition of 20, 35, and 50% water

Water Added (%)[a]	Dog Food Brand							
	#1	#2	#3	#4	#5	#6	#7	#8
20	0.94[b]	0.96	0.94	0.97	0.95	0.97	0.93	0.92
35	0.98	0.97	0.98	0.99	0.99	0.99	0.97	0.95
50	>0.99	>0.99	>0.99	>0.99	>0.99	>0.99	>0.99	>0.99

[a]Proportion of dry weight
[b]Mean of two samples

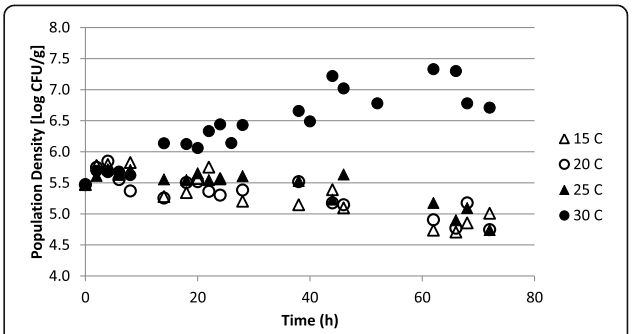

Fig. 2 Growth of *S.*Typhimurium CVM98 and *S.* Enteritidis KPL13076 in brand #4, rehydrated to 35% moisture and incubated at 15, 20, 25, and 30 °C

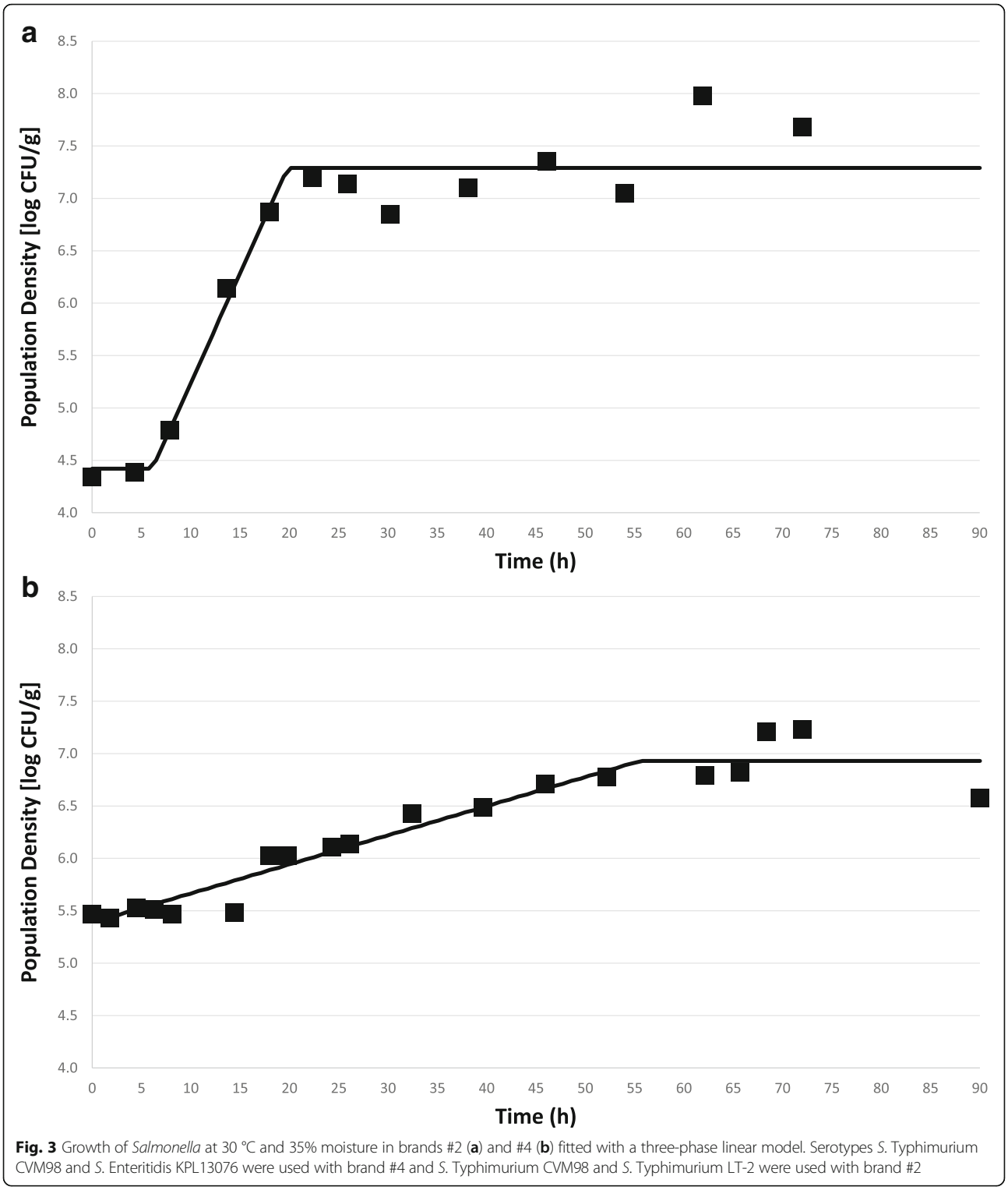

Fig. 3 Growth of *Salmonella* at 30 °C and 35% moisture in brands #2 (**a**) and #4 (**b**) fitted with a three-phase linear model. Serotypes *S*. Typhimurium CVM98 and *S*. Enteritidis KPL13076 were used with brand #4 and *S*. Typhimurium CVM98 and *S*. Typhimurium LT-2 were used with brand #2

further increased to 35% and 50%, the a_w was closer to the optimal for *S. enterica*. At these higher moisture levels, three of the brands continued to display reductions in *Salmonella* levels (Fig. 1b, c). This suggests that these brands may have an antimicrobial ingredient that is released or activated with increased water availability. However, comparison of the ingredients labels for each of the brands did not provide any insights into the specific component(s) of the formulations that would account for the putative antimicrobial response. In general, bacterial

counts on non-selective BHIA and selective XLDA plates were similar (Fig. 1a-c), suggesting that no other microorganisms were growing when the *Salmonella* were not. This also suggests that in most instances when *S. enterica* grew, there was little injury. However, as mentioned above, this was not the case in brands #7 and #8 where *S. enterica* levels declined (Fig. 1b).

Two preliminary studies were undertaken to characterize the growth kinetics of *Salmonella* in 35% rehydrated dog food. In the first, the strong temperature effect on *S.* Typhimurium CVM98/*S.* Enteritidis KPL13076 in brand #4 was evaluated at 15, 20, 25, and 30 °C (Fig. 2). Growth was only observed at 30 °C which is consistent with the earlier results based on a 72 h incubation at 28 °C.

In the second preliminary study, the growth kinetics of *S.* Typhimurium CVM98/*S.* Typhimurium LT-2 were determined at 30 °C after rehydration of brand #2 (Fig. 3a). These conditions supported growth to levels similar to those observed with brand #2 with 72 h incubation at 28 °C (Fig. 1b). The 30 °C growth curve data for brands #2 and #4 were fitted to the three-phase linear growth model (Fig. 3a, b) to generate commonly used growth kinetics metrics (Table 3). While the lag phase duration was similar for the two brands, there was a substantial difference in the generation times. This resulted in brand #4 taking a substantially longer time to reach its maximum population density. The reason(s) for the difference in growth rates is unclear, particularly considering that brand #4 had a slightly higher a_w after rehydration (Table 2). Additional research is currently underway to explore the reasons underlying the differences noted among the brands in regard to the survival or growth of *Salmonella.*

The current study clearly demonstrates that rehydration of dry dog food may support the growth of *S. enterica* if present. The specific role that rehydration plays in outbreaks of salmonellosis among pet owners has not been considered in past outbreak investigations (CDC U.S. Centers for Disease Control and Prevention 2008, 2012; Behravesh et al. 2010), but should be in the future. Likewise, consideration of rehydration as a contributing factor in exposure assessments (Lambertini et al. 2016c) and future risk assessments may be critical for accurately estimating risks and identifying practical risk mitigation strategies. However, this could be a significant risk management challenge considering the substantial differences among brands observed in the current study.

Table 3 Growth kinetics parameters obtained by fitting the three-phase linear model to *Salmonella* growth kinetics data for brands #2 and #4, rehydrated to 35% moisture and incubated at 30 °C (see Fig. 3)

Kinetics Parameters	Brand #2	Brand #4
Lag Phase Duration	4.4 h	2.2 h
Exponential Growth Rate	0.209 Log(CFU/g)/h	0.028 Log(CFU/g)/h
Generation Time	1.4 h	10.8 h
Maximum Population Density	7.3 Log(CFU/g)	6.9 Log(CFU/g)
Time to N_{max} (TN_{max})	20 h	56 h

Conclusions

The results of this study establish that the rehydration of dry dog food with sufficient amounts of water can support the growth of *S. enterica*. Thus, if *S. enterica* is present, allowing rehydrated dog food to sit too long uneaten, particularly at warmer temperatures, is likely to increase the risk of salmonellosis for both the dog and its owners. Based on the observed lag time, the present work also indicates that these risks could be effectively managed by ensuring that uneaten food is discarded or refrigerated within approximately 2–3 h of rehydration. The data in this study also help inform a quantitative exposure assessment for *S. enterica* in dry dog food (Lambertini et al. 2016c).

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Authors' contributions

RAO was involved in the design of the study and experiment protocol, carried out laboratory experiments, data collection and drafted the manuscript. RLB was involved in the study design, data analysis, and manuscript review. EL assisted in data analysis and manuscript review. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interest.

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