ABSTRACT

Title of Thesis: EVALUATION OF THE BEHAVIOR OF *SALMONELLA ENTERICA* IN REHYDRATED DRY DOG FOODS

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Recent human salmonellosis outbreaks associated with dry dog foods have raised concern over these products as potential vehicles for *Salmonella*. In this study, different behavior (decline or growth) of *Salmonella* across twenty-six different brand dog foods that were rehydrated to a moisture content of 35% and stored at 30°C for 72 hr were characterized. Decline data were fitted with log-linear model and growth data were fitted by reparameterized Gompertz model. The distributions for the parameters in the fitted reparameterized Gompertz model were obtained. The effects of pH and water activity of rehydrated dog foods on changes in *Salmonella* levels (Log CFU/g) within 72 hr were modeled by the second order polynomial regression. The results can be implemented in the future quantitative microbial risk assessment studies. This study was useful in providing critical information regarding *Salmonella* and dog food to develop effective contamination prevention and mitigation strategies.

EVALUATION OF THE BEHAVIOR OF *SALMONELLA ENTERICA* IN REHYDRATED DRY DOG FOODS

by

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Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Master of Science
2017

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Acknowledgements

I would like to express my deepest gratitude to my advisor, Dr. Abani Pradhan, for his support, caring, patience, and guidance through the past two years. It would not be possible to complete my research without help from him.

Also, I would like to thank Dr. Robert Buchanan and Dr. Seong-Ho Lee for serving as my committee members as well as giving me invaluable advices and guidance on my research.

I would also like to thank Dr. Abhinav Mishra for his discussions and suggestions during writing my thesis. Also, I would like to appreciate the experimental help from Mr. Anthony Soc, Ms. Caroline Johnson, Ms. Yuyang Lu, and Ms. Wendy Guan. I would like to thank my colleagues and friends, Dr. Abhinav Mishra, Dr. Hao Pang, Ms. Surabhi Rani, Ms. Shraddha Karanth, Ms. Yuqing Ying, and Dr, Jinyao Chen for their suggestions and encouragements. I would like to thank my parents for their endless love and support during the past years.

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Chapter 1. Introduction

Pets play an irreplaceable role in families and in people's daily life. According to 2017-2018 APPA National Pet Owners Survey, 60.2% of households own dogs at home, which accounts for 74.9 million families in the U.S. (APPA, 2017). When feeding their dogs, it was estimated that more than 90% of dog owners choose to feed commercial dry dog foods or treats (Stull et al., 2013; Connolly et al., 2014).

Salmonella is considered as one of the leading causes of human illnesses and gastrointestinal diseases. In the past decade, several human salmonellosis outbreaks were associated with contaminated dry dog foods and treats (CDC, 2012). The reasons for its frequent occurrence were mainly because of intimate contacts with pets, improper sanitation procedure, and incorrect feeding practices (Fischer et al., 2007; Lambertini et al., 2016a).

Dog owners may elect to moisten commercial dry dog food by different means. For example, dog food is rewetted to increase its palatability and to achieve a desired soft texture for easy chewing. The rehydration could result in providing a favorable environment for foodborne pathogens to survive and potentially grow, which ultimately threatens dogs' and their owners' health. However, the rehydration of dog foods has not been widely discussed. Only a very few studies have been conducted on *Salmonella* behavior in rewetted dog foods. Thus, the objective of this study was to examine and characterize the behavior of *Salmonella* on different brands of commercial dry dog food under the condition of being rehydrated to a moisture level of 35% and stored under 30°C for 72 hr. The results from this study can be

applied to the future quantitative microbial risk assessments. The study would be helpful in developing prevention strategies related to *Salmonella* in dog food.

Chapter 2. Literature Review

2.1 *Salmonella* and salmonellosis

Salmonella is a small, rod-shaped, non-spore forming, gram-negative bacterium of the Enterobacteriaceae family (U.S. FDA, 2012). They usually used glucose as their nutrition source. The optimum pH requirement for them to grow is around neutral, which varies from 6.6 to 8.2. They can grow at the temperature ranging from 4 to 47°C, with optimal growth occurring at around 37°C. They can rarely grow under the situation when water activity is lower than 0.90 (Jay et al., 2012).

Some non-typhoidal strains of *Salmonella enterica* are highly pathogenic and typically cause illness and gastrointestinal disease in human (Rabsch et al., 2001). The infectious dose differs by serotypes. Typically, the infectious dose is around 10^3 bacilli for non-typhoidal salmonellosis (Ryan and Ray, 2004; Bronze and Greenfield, 2005). People who are elders (>60 years), young children, immuno-compromised individuals, or patients suffering from various diseases are more likely to be infected at a lower doses, which can be a single cell in some extreme cases (U.S. FDA, 2012).

According to the Centers for Disease Control and Prevention (CDC), every year in the United States, it was estimated that 1,000,000 foodborne illnesses, 19,000 hospitalizations and 380 deaths were caused by *Salmonella enterica* (CDC, 2016). Contact with animals or animal products was one of the leading causes of human salmonellosis (Behravesh et al., 2010; Hale et al., 2012; Stull et al., 2013). Based on a 2011 review study, 9% of human salmonellosis cases were caused by direct contact with animals (Hoelzer et al., 2011). On average, at least 1% of salmonellosis cases

reported annually is associated with contact with companion animals (Stehr-Green and Schantz, 1987; Guardabassi et al., 2004). A study in 2012 indicated that based on all the 2,058 samples collected, including animal feeds, pet food/treats and pet supplements, 12.5% of the samples tested positive for *Salmonella enterica* (Li et al., 2012). Based on these evidences, factors including contact infected animals or pets, and improper handling of pet food could be the reasons for contracting human salmonellosis.

Dogs have a relatively high frequency of salmonellosis (Moran, 1961; Morse et al., 1976). Studies showed that the prevalence of isolating *Salmonella* from dogs ranged from 1% to 36% (Finley et al., 2006), with various serotypes isolated (Morse et al., 1976). Typically, dogs showed no symptoms of being infected by *Salmonella*, which means they served as asymptomatic carriers, and could transmit microorganisms to human (Enriquez et al., 2001). In some worst cases, they may suffer the symptoms including fever, malaise, vomiting, abdominal pain, weight loss, cough, nasal hemorrhages, or diarrhea (Morse et al., 1976; Finley et al., 2006). Cardiovascular collapse and shock may also occur depending on specific situations, such as host health condition and virulence of the strains. (Greene, 1984; Finley et al., 2006). Once being infected, dogs can shed *Salmonella* for six weeks or more (Morse et al., 1976; Sanchez et al., 2002).

Over the years, many pet foods recalls occurred due to the potential adulteration of *Salmonella enterica*. In 2013, the New Hampshire Department of Health and Human Services (NH DHHS) announced a recall of Joey's Jerky brand Chicken Jerky due to the possible contamination of *Salmonella*. A total of twenty-one

people were sick possibly due to *Salmonella* infections (NH DHHS, 2013). Besides, several multi-state human salmonellosis outbreaks were associated with contaminated dog food and treats (Table 1).

Table 1. Recent *Salmonella* outbreaks associated with pet food and treats

Year	Strains	Commodity	Regions	Reference
1999	*S.* Infantis	Pig ear dog treats	Alberta, Canada	(Clark *et al.*, 2001)
2002	*S.* Newport	Pet treats containing dried beef	Calgary, Alberta, Canada,	(Pitout *et al.*, 2003)
2005	*S.* Thompson, *S.* Cerro, *S.* Meleagridis	Pet treats made from of beef or seafood	Alberta, BC, Washington	(CDC, 2006) (CDC, 2008a)
2006	*S.* Schwarzengrund	Dry pet food	U.S. (21 states)	(CDC, 2008)
2012	*S.* Infantis	Dog food	Canada (21 states)	(Imanishi *et al.*, 2014)

In 1999, 12 cases of *Salmonella enterica* serotype Infantis were reported and the strain originated from contaminated pig ear dog treats. Investigation also indicated 18.5% of infected dogs were symptomatic (Clark et al., 2001; Lambertini et al., 2016a). In 2006, a total of 79 cases of *Salmonella* Schwarzengrund human infection were reported in 21 states in the U.S. and the strain was isolated from a dry dog food (CDC, 2008a,b). Another multistate *Salmonella* Infantis outbreak occurred in April 2012, which caused 53 human illnesses across 21 states and 2 provinces in Canada. The pathogen was traced back to an unopened dry dog food manufactured in a factory in South Carolina in the U.S. (Imanishi et al., 2014). Such outbreaks and product recalls caused a tremendous impact on the food industry, and human and dogs' health (Hoelzer et al., 2011). Therefore, attention has been raised to examine whether and how *Salmonella* can survive in dry dog food.

2.2 Transmission of *Salmonella* between humans and dogs

Among the new-emerging human infections, around 75% of cases were estimated to be zoonotic (Taylor et al., 2001; Day et al., 2012). For those infected companion dogs that do not receive enough medical care or examinations, they can act as reservoirs for a variety of zoonosis. The poor veterinary medical care system can increase the risk of transmitting infectious diseases from animals to humans. The dissemination of such diseases ultimately threatens human's health (Day et al., 2012).

The transmission of human salmonellosis from dogs can be summarized by two main routes. One is direct contact with infected dogs. The second is indirect contact with fecal-contaminated environment or foods.

First, pathogen transmission can occur when directly touching animals' skin, mucous membranes, or body fluids, for example, by animal bites and scratches (Mani and Maguire, 2009; Stull et al., 2013). The closer association between companion dogs and households may increase the possibility of direct touching (Day et al., 2012). Since more households consider dogs to be their family members, closer physical contact including touching, licking or petting occurs more frequently than in the past (Guardabassi et al., 2004).

The other primary mode of transmission is fecal-oral transmission. Where staying in a fecal-contaminated environment, pets can spread the zoonotic microorganisms on a large-scale. For example, contact or playing with pets in public setting areas may increase the risk of disease dissemination between humans and pets via shared facilities (CDC, 2005). Such public setting areas include petting zoos, animal playgrounds, and rest and feeding areas. Research showed the prevalence of

Salmonella was higher in the summer and fall months when there were a large amount of animal traveling exhibitions and petting zoos scheduled (CDC, 2005). Inadequate separation between animal activity areas and human food-consumption areas can cause cross contaminations (Crump et al., 2003; CDC, 2005). Moreover, improper defecation from dogs may increase environmental contamination (Cinquepalmi et al., 2013). The presence of dog stools in a shared environment with humans, or at a river site will increase the risk of fecal-contaminations and induce the spread of zoonotic diseases. Specifically, studies indicated that some antibiotic-resistant bacteria and multidrug-resistant bacteria were more often found in pets and their stool samples (Rodrigues et al., 2002). Some multidrug-resistance bacteria include *Salmonella* Typhimurium and *Salmonella* Newport (Zhao et al., 2003).

Moreover, the spread of zoonotic microorganisms between animals and humans can happen by ingestion of fecal-contaminated food, drink or materials in a shared environment (D ’aoust, 1978; Stull et al., 2013). Household environments could serve as one the reservoirs for *Salmonella* persistence (Rice et al., 2003). Kitchen is a good example and was usually where contamination takes place (Fischer et al., 2007; Lambertini et al., 2016a). People tend to prepare their own food and dog food at the same place without paying enough attention to cautions. Additionally, studies demonstrated that *Salmonella* was capable of surviving in feeding bowls for a long period of time, even after bowls were washed with soap and disinfected (Weese and Rousseau, 2006; Laflamme et al., 2008). Several spots, such as countertops and refrigerators, from a home environment were tested positive for *Salmonella*. (Schutze

et al., 1999). Without proper sanitation practices, cross contamination may result in human salmonellosis.

The most vulnerable population to such transmitted disease are usually immuno-compromised individuals, patients with other diseases, elders (>60 years), neonates, and young children (Mani and Maguire, 2009). Young children especially tend to spend more time and have much closer contact with companion dogs directly or indirectly.

2.3 Dog ownership and feeding practices

By the end of 2012, the American Veterinary Medical Association (AVMA) estimated that in the United States, more than 43.0 million households owned dogs (AVMA, 2013). According to a most recent 2017-2018 APPA National Pet Owners Survey, 60.2% of households raise dogs at home, which accounts for 74.9 million families in the U.S. (APPA, 2017). The number and percentage of the families owning pets have continued to increase. Interestingly, it was estimated that the average number of dogs owned per household was 1.6, which means people tend to raise more than one pet at home (AVMA, 2013).

When feeding dogs, most households choose to feed them with commercial dry dog food (Stull et al., 2013; Connolly et al., 2014; Oni et al., 2016). A study conducted in Ontario, Canada showed that among 264 cases surveyed, 92.4% of people fed their dogs with commercial canned/dry food, and 59.1% of people choose commercial processed pet treats (Stull et al., 2013). Another study investigated more than 2,000 dog breeders in the U.S. and Canada about their feeding practice. Their

results also confirmed that the majority of households gave a commercial kibble (dry) diet to their dogs (Connolly et al., 2014).

Dog breeders tend to spend great amounts of time having physical contact and sharing a common eating environment with their dogs (Laflamme et al., 2008). A survey conducted in the U.S. and Australia pointed out that petting or cuddling with dogs was the top common activity shared by dog owners and their dogs, which accounted for the 94.8% population investigated in the study. In addition to that, people also watched their dogs eat (31.5%), and/or eat together with dogs (22.2%). Particularly, children and infants would directly contact with the dog foods and might accidently ingest them (Lambertini et al., 2016a).

2.4 Pathogen control practices in dog food

Dry pet food/treats are considered a type of food product with a low moisture, low water activity (a_w), and a complex composition (Lambertini et al., 2016a). The finalized product usually has an a_w of 0.65 or lower, and a moisture content of 12% or less (Carrión and Thompson, 2014; Lambertini et al., 2016a). Dry dog food/treats are also considered as high-fat food products (Carrión and Thompson, 2014). Dog food kibbles are normally coated with fat contents to increase the palatability (Crane et al., 2000).

At such low moisture and low a_w level, most microorganisms including bacteria, molds, and yeast should not be able to survive. However, based on some reported outbreaks, some pathogens, including *Salmonella*, existed in dry dog food/treats and were able to survive for a long period of time (Lambertini et al.,

2016b). The possible reasons for pathogen contaminations include pre-contaminated ingredients, pathogens hiding in the fat contents of food products (Carrión and Thompson, 2014), poor hygiene practices, and imperfect equipment requirements (Carrasco et al., 2012; Finn et al., 2013). In addition to dry dog food, such pathogen can also persist in the manufacturing environments (GMA, 2009). Such capability could result in the long-term contamination of processing plants, atmosphere, floors, and production facilities (GMA, 2010; Carrión and Thompson, 2014). Thus, to practically control the incidence of pathogens in animal feeds, including dog food, has become one of the top issues in food industry.

In general, the practices of controlling *Salmonella* in dog food can be followed by three major principles, namely, avoiding introducing contaminations to the facility, inhibiting the microbial growth, and applying practices to kill the pathogens (Jones, 2011). For instance, controlling the dusts can help avoiding bringing contaminants to the processing plant. It acts as the very initial, but crucial step to ensuring the safety of the end products, because dusts is considered as one of the major sources of *Salmonella* contaminations in the feed manufacturing environment (Butcher and Miles, 2011). Secondly, to inhibit the microbial growth, applying drying process and using preservatives are two commonly used methods. Drying process is used to reduce moisture content of food product in order to ensure no or little microbial growth (Jones, 2011). In addition to drying, food preservatives were intentionally added to dog food to inhibit the growth of pathogens. Organic acids were widely used to animal feeds to control the level of *Salmonella*. Such organic acid include formic acids, sorbic acids, propionic acids, and formaldehyde

(Ha *et al.*, 2000). One study indicated that with the presence of salt, the effectiveness of potassium sorbate, a salt form of sorbic acid, can be enhanced (Larocco and Martin, 1981). Additionally, more recently, people started to use pathogen bacteriophage in pet feed production to destroy the targeted pathogens (Heyse et al., 2015; Soffer et al., 2016). Although there is no specific regulation on the usage of additives in dog food, all the additives should be either the substances that are Generally Recognized as Safe (GRAS) or they should be approved by the FDA in terms of the usage and dosage. Title 21 Code of Federal Regulations, Parts 570, 571, and 573 are the regulations on general food preservatives that can be used in animal feeding. Thirdly, in terms of killing pathogens, extrusion process has been widely applied in the production of dry dog food kibbles (Lambertini et al., 2016a). Extrusion is a common pathogen elimination step and is served as a critical control point. During the extrusion process, a treatment combining high temperature (100 to 200°C) and high pressure (34 to 37 atm) will apply to a food product, which will create a food sterilization process (Zicker, 2008). Studies confirmed the efficiency of applying extrusion to reduce the level of *Salmonella* Typhimurium in dry feed. The level of *Salmonella* was showed to decrease by more than 8 Log CFU/g at 83°C and 103°C (Okelo et al., 2006). In addition, the efficacy of extrusion can be enhanced by applying higher temperature, such as an increase to 115°C to 125°C (Fancher et al., 1996; Jones, 2011).

2.5 *Salmonella* survival in dry dog food

Dry food products are not usually considered as major sources of foodborne pathogens to grow and even to survive, due to the low water activity and low moisture level. Normally, a_w of 0.6 is the minimum requirement for most microorganisms to survive, and a_w of 0.87-0.91 is necessary for them to grow (Beuchat et al., 2013; Finn et al., 2013). Low water activity food products are considered the food with an a_w between 0.65 and 0.80 (Farkas, 2007; Lambertini et al., 2016b), for example cereals, nuts, chocolate, cocoa powder, powdered infant formula and dry pet food. (Beuchat et al., 2013; Finn et al., 2013; Lambertini et al., 2016b; Oni et al., 2016). The a_w value designed for a food product depends on different factors including ingredient compositions, pH, temperature, and storage/processing conditions, to name a few.

Salmonella has the ability of surviving in dry food products for weeks, months, and years, though the minimal a_w for their growth is 0.9. Those dry food products include cereals (Abushelaibi et al., 2003), wheat (Crumrine and Foltz, 1969), almonds (Harris et al., 2012; Kimber et al., 2012), cocoa powder (Juven et al., 1984), infant formula (Juven et al., 1984) and dry dog food/treats (Imanishi et al., 2014). Due to the complexity of the food matrix, the reason and mechanism of why *Salmonella* is able to survive in a harsh condition has not been well understood (Finn et al., 2013).

One of the relevant studies conducted by Lambertini et al. (2016b) revealed the potential risk of *Salmonella* in dry dog food. The study was to model the survival kinetics of *Salmonella* in dry dog food over approximately 600 days at room

temperature. A cocktail of 12 *Salmonella* strains isolated from previous outbreaks was inoculated on the dog foods. The results illustrated the long-term persistence of *Salmonella*. The results showed that during the 600-day experiment period, *Salmonella* suffered a 3 Log CFU/g reduction during the first 54 days with a relatively rapid decline rate. During the next 50 days, the decline rates decelerated progressively. The decline rate was further reduced from day 101 to day 570. It was noted that after this 19-month experiment, the level of *Salmonella* was stable at 2-3 Log CFU/g. The survival kinetics of *Salmonella* was then well fitted with the Weilbull model, which can be used in microbial quantitative risk assessment studies. These results inferred the risks of *Salmonella* infection on the dried dog food products that stored for almost two years under room temperature conditions.

2.6 *Salmonella* survival in rehydrated dry dog food

People may introduce water to dog foods intentionally or unintentionally by different means. In most cases, dog owners choose to add water or other liquid solution to dog food kibbles. For instance, some commercial dry premix dog foods are designed to be mixed with water and other ingredients right before feeding to the dogs (Connolly et al., 2014). Likewise, for some dog food supplements, such as probiotics/prebiotics, omega-3 fatty acids, vitamins, and minerals, they require having water dissolved first and then mixed with dog food, which is another way of rewetting dog food. The addition of these supplements becomes more common, considering more people can afford such supplements and are more willing to purchase to help their dogs meet nutrition requirements (Connolly et al., 2014).

Another situation is that pet breeders opt to add water to soften the kibbles for easier chewing, swallowing and better absorption, especially when considering some old dogs or young puppies that may have periodontal problems and imperfect digestive systems. On the other hand, there are some other ways of unintentionally introducing water to dog foods. For example, dog breeders may use a feeding bowl that is not dry enough. Or, dogs, themselves will accidentally mix foods with their drinking water or even their saliva. Thus, by all those ways, in most of cases, pet owners are unconscious of rewetting dog food before feeding. However, the scenario where dry dog foods are intentionally or accidentally rehydrated has not been completely described.

Rehydration is a crucial step that needs to be taken with more considerations. Multiple studies found the significant growth of foodborne pathogens on the rehydrated food products that usually stay at a dry condition (Deng et al., 1998; Jaquette and Beuchat, 1998; Abushelaibi et al., 2003; Richards et al., 2005; Lin and Beuchat, 2007; Lambertini et al., 2016a). A study indicated that all three bacteria, *Shigella flexneri*, *Salmonella* Enteritidis, and *Vibrio Cholerae*, were able to revive and reach to a level of 9 Log CFU/g in rehydrated infant formula after 24 hr (Wu et al., 2002). Another study also illustrated the ability of *Salmonella* to grow in an infant cereal product that was moistened by water under both 15°C and 25°C temperature conditions (Abushelaibi et al., 2003).

The recent study by Oni et al. (2016) was conducted to evaluate the potential growth of *Salmonella* in rehydrated dry dog food, which also confirmed the persistence of *Salmonella*. Specifically, a factorial study was designed on eight

brands of dog food that were rehydrated by three levels of moisture levels (20, 35, 50%) and were stored under three temperature conditions (18, 22, 28°C) for 72 hr. The results clearly illustrated that among the eight pet foods examined, when rehydrated to 20% moisture level, all dog foods inhibited the growth of *Salmonella* under all temperature levels. Whereas, the behavior of *Salmonella* varied when those pet foods were rehydrated to higher moisture levels, depending on different temperature conditions and moisture levels. Nevertheless, the study elucidated the potential risk of *Salmonella* infection associated with rehydrated dog foods (Oni et al., 2016).

No further study of characterizing *Salmonella* behavior in rehydrated dog foods was available. Thus, it is critical to further understand and characterize the behavior of *Salmonella* in rewetted dog foods, especially under a home-like feeding environment.

Chapter 3. Research Objectives

The overall goal of this project was to assess the behavior (decline or growth) of *Salmonella enterica* in rehydrated dry dog foods, and to provide useful data and information that would be helpful in developing and implementing mitigation strategies for *Salmonella* in dog food.

Specifically, the objectives of this study were to:

1) Characterize the behavior of *Salmonella enterica* in rehydrated dry dog foods, for a range of different dog food formulations with different U.S. brands, rehydrated to a moisture level of 35% and stored at warm ambient temperature of 30°C for 72 hr.

2) Fit the growth or decline curves with suitable mathematical functions for the use of future quantitative microbial risk assessments.

Chapter 4. Materials and Methods

4.1 Selection of dry dog foods

A selection of 26 dry dog food formulations with different U.S. brands and intended for dogs of various ages and health status was obtained. The acquired dog foods were stored sealed at room temperature. Due to the confidentiality agreement with the study funder, the information regarding the specific brand names and types were not provided in this study.

4.2 *Salmonella* strains and inoculum preparation

A cocktail of three *Salmonella enterica* strains including *Salmonella enterica* serovars Infantis, *Salmonella enterica* serovars Typhimurium, *Salmonella enterica* serovars Newport was used in this study. The strains were previously isolated from pet foods and pet treats. Those were obtained from the culture collection of the Department of Nutrition and Food Science at the University of Maryland, College Park.

For each of selected *Salmonella* strain, one loopful of culture was first grown by streaking on non-selective BHI (Brain Heart Infusion) Agar and selective XLD (Xylose Lysine Deoxycholate) Agar (Becton Dickinson, Sparks, MD, US). The purpose of using both non-selective and selective media was to examine if there was any contamination in the original culture. One single black colony from XLD agar was picked and enriched in 10 ml BHI broth (Becton Dickinson) at 37°C for 24 hr. After incubation, a 1.0-ml inoculum from each culture was transferred to 40 ml of BHI broth at room temperature (~25°C) for 24 hr.

Cells from each culture were harvested by centrifugation at 4800 rpm for 12 minutes at 15°C (Beckman GS-15R Centrifuge; Beckman Coulter, Brea, CA, US). The cell pellet was re-suspend in 40 ml 0.1% Buffered Peptone Water (BPW) (Becton Dickinson). After being fully mixed, a 20-µl inoculum from each individual strain was transferred to a single tube containing 40 ml 0.1% BPW, which was considered as one mixed inoculum with a cocktail of three *Salmonella* strains. The expected final concentration of this mixed inoculum was approximately 9 Log CFU/g. The mixed inoculum was used to inoculate the dog foods as described in section 4.4 below.

4.3 Measurements of moisture content, pH, and water activity

Moisture content (MC), pH, and water activity (a_w) of each brand of dry dog food were measured when opening a new product bag.

4.3.1 Measurement of moisture content

For each brand of dog foods, three 30-g portions of original dog food samples were weighed in three glass containers. For each sample, net weight and initial gross weight including the sample and container were recorded. The prepared samples were placed in the oven (1300U Gravity Convection Utility Oven, VWR, Radnor, PA, US) at 110°C for 24 hr to dry out the samples. Samples were taken out and final gross weights were weighed immediately. Initial moisture content (MC_i%) of original dog food was calculated as the gross weight difference before and after the oven treatment divided by the sample net weight.

After obtaining initial MC_i% of original dog food, the amount of liquid inoculum needed to add to a 15-g original dog food sample to reach 35% moisture level was calculated. The formula showed as follows:

$$V = (35\% - MC_i\%) \times 15$$

Where, V = Amount of liquid inoculum needed to add to a 15-g original dog food sample to reach a final moisture level of 35% (ml).

MC_i% = Initial moisture content of original dog food

4.3.2 Measurement of pH

To carry out the pH test, small portions of dog food kibbles were taken out from the original package and were pulverized by using a small wooden mallet. Five ml of distilled deionized water was added to 2-g pulverized dog food in a small beaker (1:2.5 g/v). A glass rod was used to mix the sample for 1 min. The pH value of the mixture was measured with a pH meter (Accumet Basic AB15 pH meter, Liquid-Filled Mercury-Free pH/ATC Epoxy Body Combination Electrodes, Fisher Scientific, Waltham, MA, US).

4.3.3 Measurement of water activity

The a_w measurements were conducted on the dog food before and after rehydration. To measure the a_w of the dog food before rehydration, a small portion of kibbles (~5 pieces) were taken out for immediate a_w measurement upon opening a new package. The second a_w measurement was performed on dog food after

rehydration. To obtain the rehydrated sample, a 15-g original dog food sample was placed in a small sample container with a lid. The appropriate amount of distilled deionized water was added to rehydrate the sample to 35% moisture content as calculated in previous section 4.3.1. Rehydrated sample was shaken for 30 s to reach a homogenous distribution of the added liquid and stayed sealed for 30 min to ensure liquid was fully absorbed. To carry out the measurements, a portion of five kibble pieces was placed into the chamber of a calibrated water activity meter (Novasina IC-500, AW-LAB SET H, Switzerland).

4.4 Dog food preparation and inoculation

On the basis of dog food label, the ingredient compositions were obtained from the ingredient list and the nutritional contents were obtained from guaranteed analysis section. For each brand of dog food, portions (~15 g) of kibble pieces were weighed and transferred into a small plastic container which was considered as one sample. Thirty-six (36) samples, including three negative control samples, were prepared for each brand of dog food examination. When there was an assortment of kibbles within a brand, kibble pieces were distributed as uniformly as possible to obtain the same kibble distribution as possible in each sample container.

To inoculate the dog food samples, based on the calculation described in section 3.4.1 above, the corresponding amount of prepared mixed *Salmonella* inoculum was inoculated to individual sample, which also resulted in rehydrating the sample to 35% moisture level. For three dog food control samples, instead of using the mixed inoculum, same amount of 0.1% BPW was used in inoculation. All the

samples were shaken for 30 s to ensure a homogeneous distribution and were stored under 30°C during the duration of the experiential period. The inoculation step was considered as the beginning of *Salmonella* growth/decline trial (0 hr). The 0-hr samples were processed immediately after inoculation step.

4.5 Sampling and enumeration

Throughout the 72-hr experiment period, 8-10 sampling processes were conducted periodically to characterize the behavior of *Salmonella*. Three dog food control samples were performed at the beginning, middle, and end of the 72-hr experiment to ensure there was no contamination of *Salmonella* from external sources and also to allow examining any background micro flora presented. Triplicate samples were performed at the same time to represent data points for one sampling time point. Each 15-g dog food sample was transferred into a WhirlPak bag (Nasco, For Atkinson, US) and mixed with 150 ml of sterile 0.1% BPW. Samples were then homogenized in a stomach machine for 3 min at intermediate speed (Stomacher 400 Lab Blender, Seward, Thetford, UK). For the initial three 0-hr samples, they were soaked for 15 min to soften the kibbles before stomaching. After stomaching process, samples were allowed to sit for 3 min to settle the food matrix. Around 8-10 ml solutions from the stomach bag was pipetted to a 15-ml tube. After another 10 min to allow further settling of homogenized kibble, the supernatant was used to make serial dilutions and was considered as 10^{-1} dilution level. Serial dilutions were then plated onto both BHI agar and XLD agar in duplicates using an Eddy Jet spiral plater (Neu-Tec Group Inc., Farmingdale, NY, US). Plates were incubated at 37°C for 24 hr.

Colonies enumeration was performed after 24-hr incubation by using an Economy Automatic Colony Counter (Flash & go, Neu-Tec Group Inc.). The reasons for using two different culture media were to examine if there was any contamination along the experiments and to provide a mean of measuring the degree of injury. The expected initial concentration for the inoculated dog food sample was 5 Log CFU/g, which would be able to examine either growth or decline of *Salmonella* in 72 hr.

Salmonella counts on both culture media were log-transformed to Log CFU/g and plotted on the time series graphs. In this study, the growth or decline of *Salmonella* was defined as follows: decline, if $N_{final} - N_0 < 0$; limited growth, if $N_{final} - N_0 \leq 2$, but > 0; and substantial growth if $N_{final} - N_0 > 2$, where, N_0 = Initial population density (Log CFU/g), and N_{final} = Population density at 72 hr (Log CFU/g). For some cases where the population density at 72 hr was not taken (Appendix A), the final population level was considered as N_{final}.

4.6 Mathematical model fitting

A total of 52 growth/decline kinetics curves were fitted to mathematical models by using GraphPad Prism (version 7.02, GraphPad Software, San Diego, CA, US). Log-linear model was selected to fit decline kinetics curves (Juneja and Sofos, 2001; Chen, 2007) and inactivation rates were recorded. The equation was showed as follows:

$$N_t = N_o - k \times t$$

Where: k = Inactivation rate (Log CFU/g/hr)

N_t = Population density at time t (Log CFU/g)

N_o = Initial population density (Log CFU/g)

t = Time (hr)

Growth kinetics were fitted with three commonly used growth models including reparameterized Gompertz, Baranyi, and three-phase linear model (Buchanan *et al.*, 1997). Reparameterized Gompertz model was selected on the basis of the best goodness of fit. The equation for reparameterized Gompertz model was expressed as follows (Zwietering *et al.*, 1990):

$$N_t = N_o + N_{max} (\exp[-\exp[(\mu_{max} \times e/N_{max})(\lambda - t) + 1]\}).$$

Where, N_{max} = Population density at stationary phase (Log CFU/g)

μ_{max} = Maximum specific growth rate (Log CFU/g/hr)

λ = Duration of lag phase (hr)

N_t = Population density at time t (Log CFU/g)

N_0 = Initial population density (Log CFU/g)

t = Time (hr)

The distribution of the three parameters (N_{max}, μ_{max}, λ) of the fitted reparameterized Gompertz model was obtained by using @Risk software (Version 7.5.0, Palisades Corporation, Ithaca, US). Akaike information criterion (AIC) value was used to evaluate appropriateness of the fit. The fitted distribution with the smallest AIC was selected.

Polynomial regression model that best described the effect of pH and a_w of rehydrated dog food on the overall population density changes was modeled. Based on the date results from two different culture media, both 1st and 2nd order of polynomial regression were fitted in MATLAB software (The MathWorks, Natick,

MA, version 2016b). The dependent variable was the *Salmonella* population density change (Log CFU/g) in 72 hr. Two independent parameters included in the regression model were the pH and a_w of rehydrated dog foods. A 2nd order (quadratic) polynomial regression showed the better fit, as compared to the 1st order equation. The equation was expressed as follows:

$$\Delta \text{ Population} = \beta_0 + \beta_1 \times a_w + \beta_2 \times pH + \beta_3 \times a_w^2 + \beta_4 \times pH^2 + \beta_5 \times a_w \times pH$$

Chapter 5. Results and Discussion

5.1 Dog food parameters

Among the twenty-six (26) brands of different commercial dog food examined in this study, the parameters for each brand of dog food including its pH, initial moisture content (MC_i%), and water activity (a_w) before and after rehydration were summarized (Table 2). The pH values for these 26 brands of dog food were ranging from 4.71 to 6.03. A food product with a pH level of 4.71 provided a relatively acidic environment, as compared to the one with a pH of 6.03. The water activity for dry dog foods was ranging from 0.28 to 0.69. The water activity after rehydration approached 0.95 with a standard deviation of 0.02 and varied from 0.91 to 1.00.

Based on the guaranteed analysis displayed on dog food package, the information including the minimum level of protein and fat, and the maximum level of fiber were collected (Table 4.). The averaged levels of protein, fat and fiber content found in 26 brands of dog food were 24.1%, 12.5%, and 4.3%, respectively.

The formulations varied across different brands of dog food. The ingredient compositions are listed in a descending order by weight. The major ingredients were energy-sourced meals that included meat-based meal (e.g., beef, chicken, lamb, and salmon), soybean-based meal, and/or whole grain corn-based meal. The minor ingredients that presented in dog foods were vitamins, minerals, and food additives. Food additives included coloring and flavoring agents, food stabilizers, preservatives, and some supplements such as anti-oxidants and probiotics.

Table 2. Summary of pH, initial moisture content (MC_i), and water activity (a_w) for 26 brands of dog food

Brand #	pH	MC_i (%)	a_w	
			Non-rehydrated	Rehydrated
1	5.46	8.0	0.44	0.95
2	5.46	8.6	0.44	0.97
3	5.58	10.0	0.54	0.99
4	5.44	8.0	0.43	0.97
5	5.04	8.5	0.50	0.95
6	4.83	12.4	0.69	0.94
7	5.54	9.7	0.54	0.95
8	5.20	7.7	0.44	0.93
9	5.78	8.5	0.47	0.96
10	5.66	7.9	0.47	0.96
11	5.58	8.1	0.50	0.95
12	5.38	7.4	0.46	0.91
13	5.48	8.2	0.51	0.93
14	5.05	15.8	0.68	0.94
15	5.47	7.9	0.48	0.96
16	6.03	8.7	0.47	0.93
17	5.08	13.1	0.67	0.94
18	4.71	7.4	0.43	0.98
20	5.50	7.6	0.44	0.99
21	5.65	6.8	0.41	0.98
23	5.89	7.2	0.36	0.98
26	5.83	5.9	0.28	0.99
27	5.37	8.8	0.42	0.96
28	5.49	8.1	0.58	1.00
29	5.48	6.3	0.38	0.98
30	5.77	7.9	0.48	0.97

Based on the ingredient lists, the presences of different types of preservatives in dog foods are summarized (Table 3). The preservatives included in dog foods can be categorized into synthetic chemicals and natural compounds. Synthetic chemical preservatives found in these dog foods included citric acid, sodium bisulfate, butylated hydroxyl anisol (BHA), propylene glycol, sorbic acid and potassium sorbate, and calcium propionate. The natural compounds found were ascorbic acid (vitamin C), and mixed tocopherols (vitamin E), garlic oil, and rosemary extracts. Mixed tocopherols was used widely being found 19 out of 26 brands of dog food. It was added to dog foods to preserve fat contents, such as animal fats, fatty acids, and fish oil. Yet, propylene glycol and sorbic acid/salt were only found in brand #6, #14, and #17. Calcium propionate was only used in brand #6. Ethoxyquin which is a controversial preservative and usually presents in dog food (AAFCO, 2014). It was not found in any of dog food brands examined in the study.

Table 3. The presence of the preservatives used in 26 brands of dog food

Brand #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	20	21	23	26	27	28	29	30
Mixed tocopherols	x				x	x	x	x		x	x	x	x		x		x		x	x	x	x	x	x	x	x
Rosemary extract											x	x	x						x			x	x	x	x	x
Citric acid	x	x	x						x				x			x			x					x		
Sodium bisulfate				x	x	x	x	x	x							x			x							
Ascorbic acid	x									x					x		x		x			x		x		
BHA	x	x	x						x					x		x	x									
Garlic Oil				x	x	x	x	x																		
Propylene glycol						x								x			x									
Sorbic acid/ Potassium Sorbate						x								x			x									
Calcium propionate						x																				

Table 4. Percentages of crude protein, fat and fiber contents in 26 brands of dog food based on the guaranteed analysis on label

Brand #	1	2	3	4	5	6	7	8	9	10	11	12	13
Crude protein (%)	27.0	21.0	22	21	27	25	28	18	21	27	25	28	28
Crude fat (%)	11.0	9.0	10	10	12	10	16	9.5	10	15	14	17	16.25
Crude fiber (%)	3.0	4.0	4	4.5	5	4	3	6	4.5	N/A	4	4	5

Brand #	14	15	16	17	18	20	21	23	26	27	28	29	30
Crude protein (%)	19	20	20	25	16	22	31	25	21	25	28	26	30
Crude fat (%)	10	13	8	10	7	10	20	11	12	16	20	13	15
Crude fiber (%)	4	7	4.5	4	4	N/A	3	4	4.5	5	3.3	4.5	3.5

5.2 Changes in *Salmonella* population density over 72 hr in rehydrated dry dog food

Based on the experimental results, different behaviors of *Salmonella* were observed on the dog foods that were rehydrated to a moisture level of 35% and stored at 30°C for 72 hr. The summary of overall *Salmonella* population density changes from 0 to 72 hr across 26 dog foods was provided in Figure 1. The time series plots including the data points from both culture media were provided in Appendix A.

Among the 26 inoculated dog foods examined, *Salmonella* declined in four dog food brands during the experimental time period. With the initial concentration of approximately 5 Log CFU/g, the level of *Salmonella* decreased by 0.56 to 3.71 Log CFU/g during 72 hr incubation. In one extreme case of brand #6, *Salmonella* was not able to detect after 48 hr on BHI agar and 4 hr on XLD agar (lower limit of detection ~200 CFU/g), which inferred a strong inactivation effect.

On the other hand, *Salmonella* either remained unchanged or grew on the other 22 brands of dog food. *Salmonella* showed limited growth on eight out of these 22 brands, while other 14 brands supported substantial growth of *Salmonella* within 72 hr. Some of the greatest population increases were observed on brand #23, #26, and #28 on which *Salmonella* levels increased by approximately 4 Log CFU/g during 72 hr incubation.

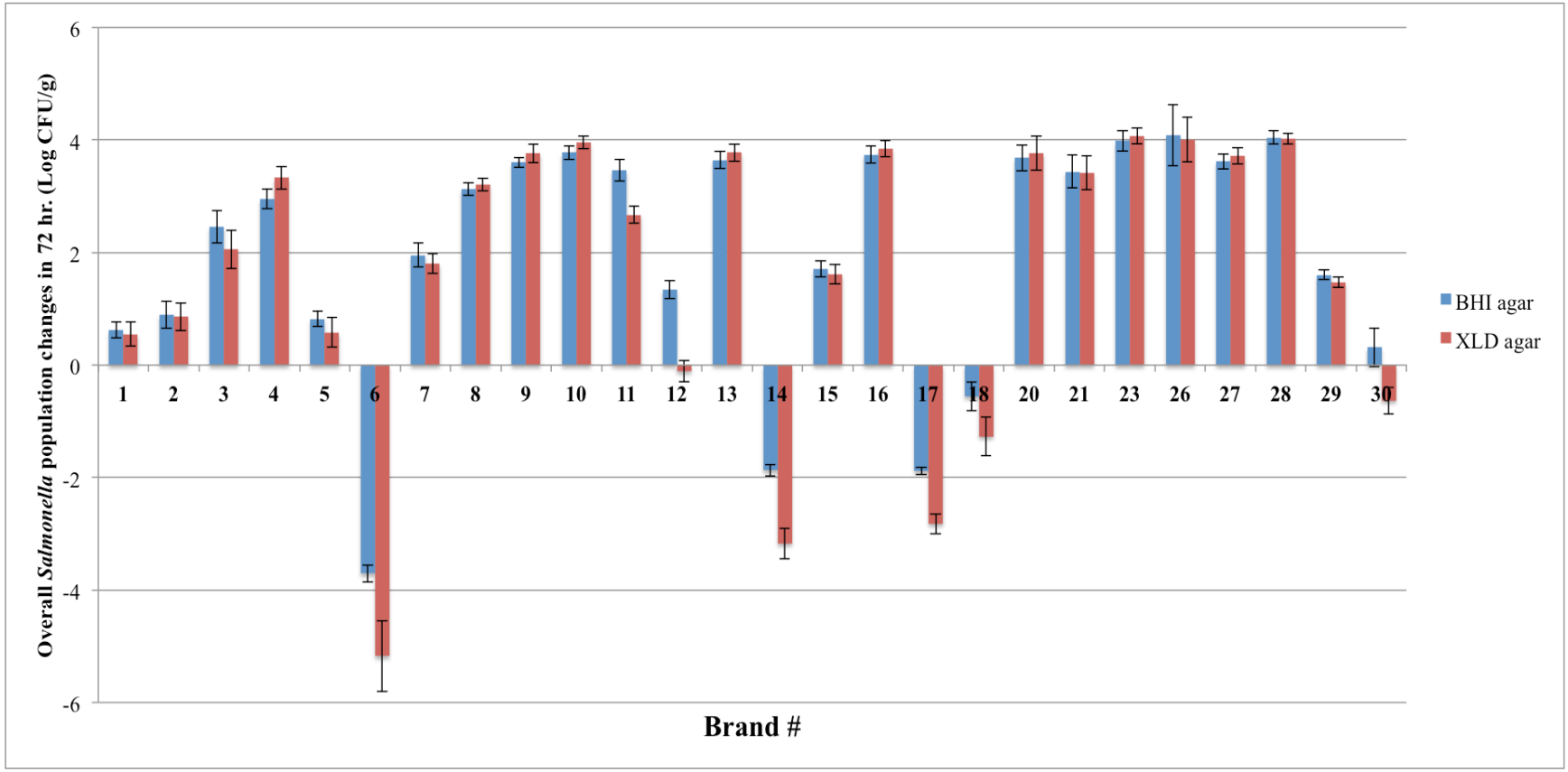


Figure 1. Comparison of *Salmonella* population density changes (Log CFU/g) in 72 hr across 26 brands of dog food. (Brand #19, #22, #24, and #25 were missing from the figure due to the unavailability of these four brands of dog food initially planned for experiments.)

Based on four decline examples, it was observed that starting from an approximately same initial population density, a more rapid decline rate was observed based on the data points from XLD agar, as compared to those from BHI agar. In other words, the number of *Salmonella* counts from XLD agar was fewer than that from BHI agar. Those observations could be explained by cell injury. Because of unfavorable growing environment, *Salmonella* got injured and injured cells could recover on non-selective media (BHI agar), but they were not able to revive on selective media (XLD agar) due to the presence of selective agents and limited nutrients. The unfavorable growing environment could be due to nutrients unavailability, the presence of antibacterial agents, and/or exposure to low-pH and low-a_w stress. Based on the calculation on the raw data (not provided here), the maximum percentage of injured cells on brand #6, #14, #17 and #18 were equated to 100%, 80.7%, 85.9% and 81.5%, respectively. Similarly, in brand #12 and #30, the number of *Salmonella* appeared to have a slight increase in 72 hr based on BHI agar. Yet, *Salmonella* was dying based on the results from XLD agar. Those observations could also be the result of cell injury.

5.3 Mathematical model fitting

The decline kinetics curves were fitted with log-linear model and the growth kinetics curves were fitted with reparameterized Gompertz model. The figures for both fitted models were provided in Appendix B.

For all the decline cases, log-linear model was selected to fit the kinetics, because there was no obvious shoulder or tail found in all decline curves. The

estimated inactivation rates were collected, as measured by the absolute value of the slope of log-linear model (Table 5). Based on the results from BHI agar, the inactivation rates varied from 0.01 to 0.05 Log CFU/g/hr with an average of 0.028 Log CFU/g/hr. To note, based on the data points from BHI agar, the inactivation rates for *Salmonella* for brand #12 and #30 were not able to obtain, because these two kinetics curve showed a slight growth and were better fitted with reparameterized Gompertz model. Besides, the inactivation rates were not fitted with a suitable distribution, due to too small sample size.

All the growth kinetics data were fitted with reparameterized Gompertz model, Baranyi model, and three-phase linear model. After comparing the goodness of fit, R^2 and RMSE, reparameterized Gompertz model was selected to fit the best with the growth kinetics. The parameters of the fitted reparameterized Gompertz model were summarized based on the data from BHI agar (Table 6) and XLD agar (Table 7).

Table 5. Summary of the inactivation rates (k) estimated based on the data from brands where *Salmonella* levels declined

Brand #	k (BHI)		k (XLD)	
	Best fit (Log CFU/g/hr)	**Std. err.**	**Best fit (Log CFU/g/hr)**	**Std. err.**
6	0.05	<0.01	0.07	<0.01
12	N/A[a]	N/A	<0.01	<0.01
14	0.03	<0.01	0.04	<0.01
17	0.03	<0.01	0.04	<0.01
18	0.01	<0.01	0.02	<0.01
30	N/A	N/A	0.01	<0.01

[a]N/A: not applicable; based on the results from BHI agar, both kinetics for brand #12 and #30 could not be fitted with log-linear model.

Table 6. Summary of the parameters of the reparameterized Gompertz models fitted for *Salmonella* growth based on BHI agar

Brand #	N_0 (Log CFU/g)		N_{max} (Log CFU/g)		μ_{max} (Log CFU/g/hr)		λ (hr)	
	Best fit	**Std. err.**	**Best fit**	**Std. err.**	**Best fit**	**Std. err.**	**Best fit**	**Std. err.**
1	6.62	0.12	7.25	0.09	0.24	324.1	11.24	2378
2	6.82	0.22	7.71	0.10	0.14	0.11	3.64	3.18
3	5.73	0.27	8.19	0.11	0.12	0.02	0.00	1.45
4	6.03	0.16	8.98	0.07	0.23	0.04	3.27	1.37
5	5.95	0.09	6.77	0.10	0.11	0.24	9.59	4.66
7	6.69	0.20	8.64	0.07	0.18	0.04	1.78	1.94
8	5.75	0.10	8.87	0.06	0.26	0.03	5.01	0.87
9	5.67	0.08	9.28	0.04	0.42	0.04	3.01	0.38
10	5.65	0.11	9.43	0.05	0.35	0.03	3.86	0.68
11	5.52	0.16	8.98	0.11	0.18	0.02	3.82	3.82
12	5.68	0.08	7.02	0.14	0.11	0.06	20.00	3.02
13	5.61	0.12	9.26	0.09	0.22	0.03	5.28	1.10
15	5.65	0.12	7.36	0.08	0.24	0.07	4.57	1.51
16	5.67	0.14	9.41	0.05	0.44	0.04	1.41	0.61
20	5.59	0.18	9.27	0.14	0.13	0.01	5.16	2.51
21	5.67	0.18	9.11	0.22	0.14	0.03	10.36	3.81
23	5.51	0.17	9.50	0.06	0.37	0.04	1.58	0.81
26	5.16	0.53	9.25	0.15	0.21	0.03	0.00	3.42
27	5.44	0.12	9.05	0.06	0.30	0.03	3.58	0.73
28	5.19	0.11	9.23	0.03	0.31	0.01	0.71	0.55
29	5.69	0.07	7.29	0.04	0.16	0.02	4.24	0.98
30	5.60	0.30	5.92	0.15	0.02	0.05	2.18	23.94

Table 7. Summary of the parameters of the reparameterized Gompertz models fitted for *Salmonella* growth based on XLD agar

Brand #	N_0 (Log CFU/g)		N_{max} (Log CFU/g)		μ_{max} (Log CFU/g/hr)		λ (hr)	
	Best fit	**Std. err.**	**Best fit**	**Std. err.**	**Best fit**	**Std. err.**	**Best fit**	**Std. err.**
1	6.36	0.19	6.91	0.01	0.04	0.03	0.00	8.93
2	6.63	0.22	7.49	0.11	0.28	0.36	4.38	2.74
3	5.51	0.32	7.56	0.11	0.16	0.05	1.85	3.19
4	5.57	0.19	8.90	0.08	0.25	0.04	2.99	1.43
5	5.63	0.21	6.21	0.16	0.05	0.08	10.41	12.90
7	6.72	0.16	8.52	0.07	0.37	0.11	3.51	0.88
8	5.56	0.10	8.77	0.06	0.28	0.04	5.72	0.86
9	5.57	0.14	9.33	0.07	0.51	0.08	4.39	0.77
10	5.39	0.10	9.35	0.05	0.36	0.03	4.09	0.65
11	5.45	0.12	8.12	0.10	0.28	0.02	6.64	1.62
13	5.42	0.12	9.19	0.09	0.23	0.03	5.54	1.06
15	5.35	0.15	6.97	0.10	0.19	0.07	4.87	2.03
16	5.44	0.13	9.29	0.06	0.49	0.05	2.42	0.50
20	5.36	0.23	9.13	0.20	0.13	0.02	6.35	3.30
21	5.34	0.18	8.76	0.24	0.14	0.03	10.93	3.94
23	5.35	0.13	9.43	0.06	0.42	0.04	2.52	0.57
26	5.19	0.36	9.20	0.16	0.21	0.04	2.03	2.73
27	5.22	0.13	8.94	0.06	0.29	0.03	3.23	0.79
28	5.04	0.09	9.06	0.04	0.36	0.02	1.79	0.45
29	5.42	0.08	6.90	0.05	0.16	0.03	4.01	1.11

The results suggested that several dog foods supporting *Salmonella* to grow were estimated to have a high μ_{max}, and a small λ value. For instance, on brand #16, the lag phase duration was estimated only for 1.41 hr and a growth rate was as rapid as 0.44 Log CFU/g/hr. Likewise, on brand #23, *Salmonella* had a lag phase as short as 1.58 hr, and could have as quick as 0.37 Log CFU/g increase per hour. These findings suggest that dog food could expose a safety risk, because *Salmonella* may able to adjust to the environment and started to have a substantial growth in a fairly short time.

The distributions of the three parameters (N_{max}, μ_{max}, λ) in the fitted reparameterized Gompertz model were selected on the basis of the smallest AIC value (Table 8). The figures for the fitted probability distributions from two different culture media were provided (Figure 2). These results can be implemented in the future quantitative microbial risk assessment studies to characterize the growth of *Salmonella* in rehydrated dog foods.

Table 8. The distributions of the three parameters in the fitted reparameterized Gompertz models

Parameters	**BHI**	**XLD**
N_{max}	RiskTriang (5.60, 9.50, 9.50)	RiskTriang (5.84, 9.42, 9.42)
μ_{max}	RiskExtValue (0.17, 0.09)	RiskUniform (0.01, 0.53)
λ	RiskExpon (4.74, RiskShift (-0.22))	RiskExtValue (3.19, 2.08)

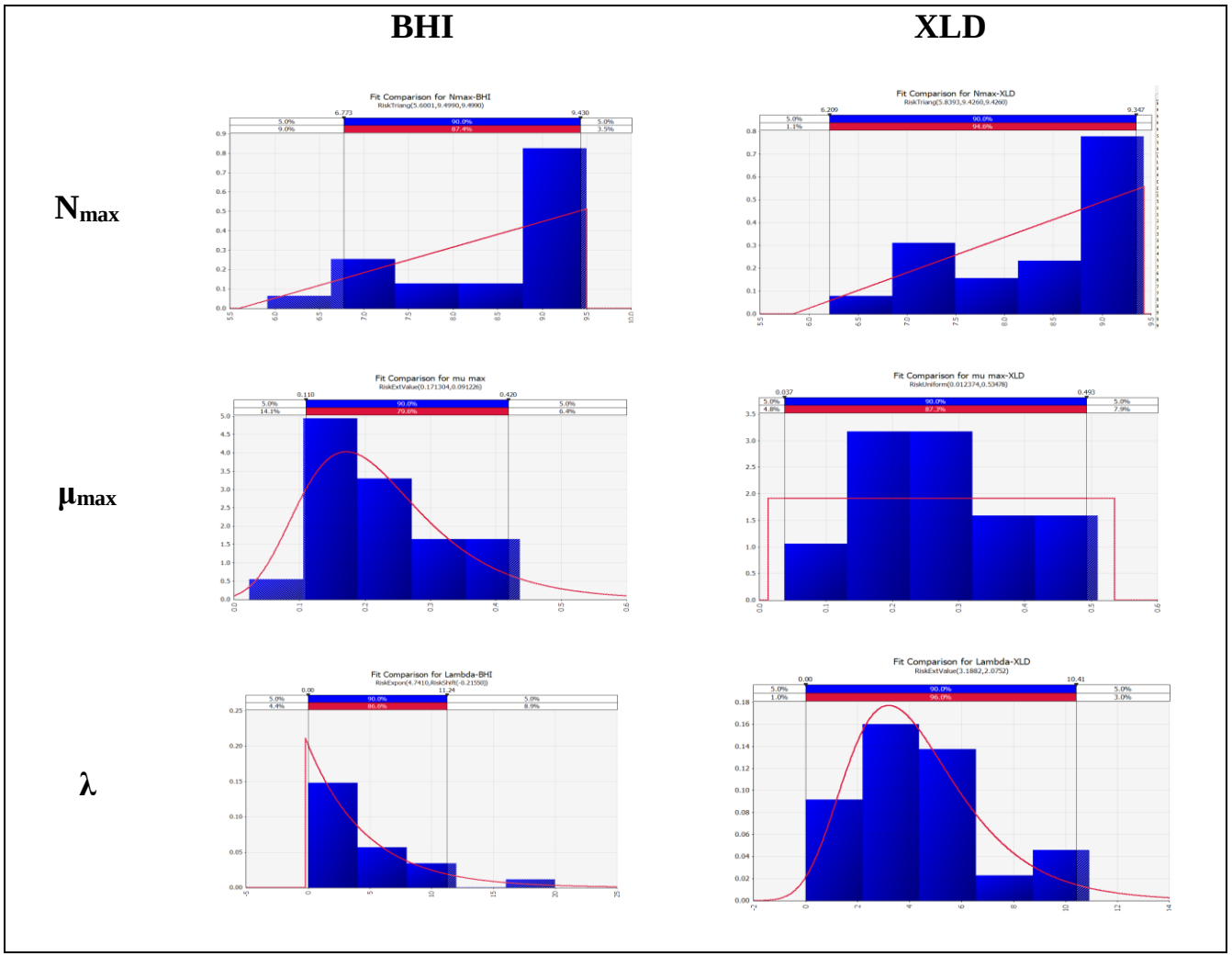


Figure 2. The distributions of the three parameters in the fitted reparameterized Gompertz models

The second order of polynomial regression was selected and was fitted better than 1st order polynomial regression model, based on a larger R^2 and a smaller error term, RMSE and SSE. Both regression models based on data points from two culture media had a R-square value of 0.67 (Figure 3). The fitted equations were expressed as:

$$\Delta \text{ Population (BHI)} = -202.8 + (373.1 \times a_w) + (133.7 \times pH) + (459.8 \times a_w^2) + (-4.05 \times pH^2) + (-89.64 \times a_w \times pH)$$

$$\Delta \text{ Population (XLD)} = -622 + (287.6 \times a_w) + (169.5 \times pH) + (188 \times a_w^2) + (-5.16 \times pH^2) + (-113.9 \times a_w \times pH)$$

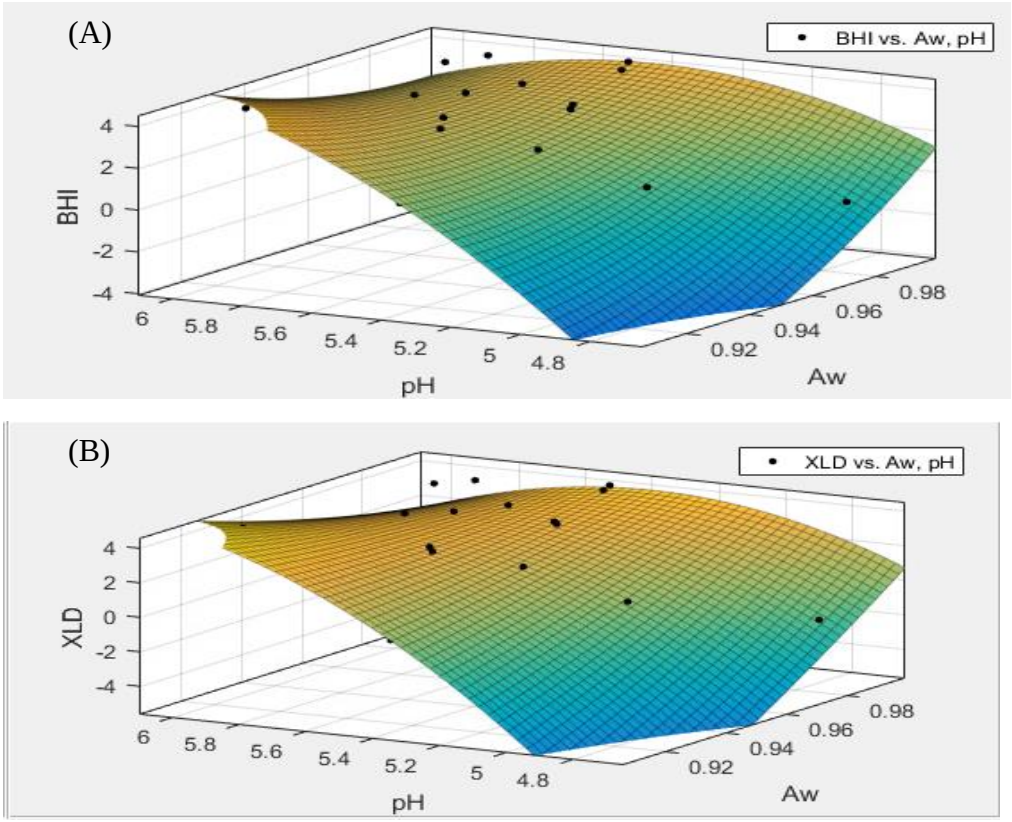


Figure 3. Second order polynomial regression figures on *Salmonella* population changes (Log CFU/g) in 72 hr vs. pH and a_w of rehydrated dog foods based on BHI (A) and XLD (B) culture media.

5.4 Discussion

By using both non-selective and selective media in this study, the results suggested that some other microorganisms, other than *Salmonella enterica*, were observed on BHI agar from some *Salmonella*-free dog food control samples (lower limit of detection ~200 CFU/g). Based on their morphologies shown on the media, some of them were more likely to be fungi, such as molds and yeasts. In some cases, molds even became visible on the surface of dog food kibbles. These observations could due to the presence of some residential microorganisms. These microorganisms that were commonly found in the dog food included *Aspergillus niger*, *Aspergillus glancus* and *Saccharomyces epidermidis* (Bueno et al., 2001). In addition, the condition where dog food was rehydrated to 35% moisture content and stored at 30°C became favorable for most microorganisms to grow. Besides, some tiny and whitish colonies were appeared on BHI agar. One of the possible reason to explain was that some probiotic strains, such as *Lactobacillus* spp., *Bifidobacterium* spp., and *Bacillus* spp. were added to the dog foods, as indicated on dog food label. Thus, further research is needed to identify the background micro flora in dog food and to examine if presence of such microbes will affect the behavior of *Salmonella* in rehydrated dog food.

A similar study was conducted by Oni et al. in 2016 that was based on a factorial design on eight brands of dog food that were rehydrated to 20%, 35% and 50% moisture level and stored under 18°C, 22°C and 28°C for 72 hr. The methodologies applied in Oni et al. study were as similar as the experimental procedures followed by this study. One of the treatment levels (35% × 28°C) used in Oni et al.'s study could

be comparable to the condition used in this study. Among the eight brands of dog food examined by Oni et al., four of them were as similar as the ones included in this study, in terms of the brand name and the manufacturer of dog food product. The results from two studies were summarized (Table 9). By comparing the results from two studies, most of the results were consistent. For instance, the results for brand #6, the same dog food labeled as #8 in Oni et al.'s study, were confirmed by both studies. Both studies indicated that this brand showed a relatively strongest inactivation effect. However, it was noted that *Salmonella* showed a limited growth on brand #5 in this study, whereas declining was observed on the same dog food, named brand #1 in Oni's study. The variation might be caused by the unknown difference of the ingredients composition present in the dog foods. Further statistical analysis can be performed to examine if the difference is significant.

Table 9. Comparisons of the results on four similar brands of dog food between this study and Oni et al. 2016 study

Study	Brand #	pH	a_w		Δ Population (Log CFU/g)*	
			Dry	Rehydrated	BHI	XLD
This study	#3	5.58	0.541	0.99	+2.46	+2.06
Oni et al.	#4	5.83	0.492	0.99	+2.6	+1.4
This study	#5	5.04	0.501	0.95	+0.82	+0.58
Oni et al.	#1	5.30	0.495	0.98	-0.2	-1.4
This study	#6	4.83	0.689	0.94	-3.71	-5.17
Oni et al.	#8	5.02	0.653	0.95	-2.5	-3.5
This study	#14	5.05	0.680	0.94	-1.87	-3.17
Oni et al.	#7	5.39	0.66	0.97	-0.7	-0.9

* Δ Population: *Salmonella* population density changes (Log CFU/g) from 0 to 72 hr

This study inferred the potential effect of pH, a_w of rehydrated dog food on *Salmonella* behavior. Based on the results from second order of polynomial

regression models, when reducing pH values and a_w of rehydrated dog food, the population density changes decreased. These finding suggested that an acidic or low a_w environment will hinder or inhibit the growth of *Salmonella*. Moreover, based on the results for dog food brand #6, #14, and #17 where *Salmonella* levels declined, they possessed relatively lower pH values, as compared with other brands. Such finding might suggest that pH effect could be predominant. Further statistical analysis can be developed to examine this hypothesis.

Another factor contributing to different behavior of *Salmonella* in rehydrated dry dog food could be different nutritional contents, especially fat contents. Several studies revealed *Salmonella* was resistant in high-fat food under thermal inactivation (Juneja and Eblen, 2000). The survivability of *Salmonella* were affected by fat contents in animal feeds (Norwegian Scientific Committee for Food Safety, 2006). Bacteria tend to hide in the fat contents and they will start to grow once the living environment becomes favorable. Thus, more studies are needed to identify the role that fat contents play in the dry and rehydrated dog food.

Furthermore, the types of preservatives used in dog food products could affect the growth of *Salmonella* in rehydrated samples. By comparing ingredient compositions for each brand, the presence of specific preservatives in dog food brand #6, #14, and #17 suggested potential antimicrobial responses. These three brands showed inhibitory effect on the survival of *Salmonella* in this study. According to Table 3., in terms of the last three preservatives, propylene glycol, sorbic acid/salt, and calcium propionate, the frequency of their presences in examined dog foods was relatively lower than the other preservatives. Propylene glycol and sorbic acid

occurred only in brand #6, #14 and #17. Calcium propionate was only found in brand #6. Thus, the potential antimicrobial effect of propylene glycol, sorbic acid and calcium propionate could be addressed. Nevertheless, to point out, brand #18 was another dog food showing the inactivation effect on *Salmonella*, but none of preservative was found based on dog food label. Further study is needed to further examine the possible mechanism of inactivating *Salmonella* in brand #18. Furthermore, research are need to identify and quantify the chemicals inside the dog foods, which will allow assessing the effect of using preservatives in reducing pathogens in dog foods.

Previous studies illustrated the strong antimicrobial effect of these three preservatives, sorbic acid, calcium propionate and propylene glycol (Erickson, 1982) and all of them showed no adverse effect to dogs and humans. For example, sorbic acid was confirmed to cause the inactivation of *Salmonella* (Park and Marth, 1972; Liewen and Marth, 1985) and the effect could be enhanced by the presence of sodium nitrate (Larocco and Martin, 1981). No adverse effect was showed on dogs if feed with the food with less than 5000 mg/kg potassium (The European Commission, 2012). Similarly, propionate acid was regarded as a strong antimicrobial and was especially efficient in *Salmonella* inhibition (Haque et al., 2009). Haque *et al.* pointed out that propionic acid had less antibiotics-resistant impact to the animals. It was widely used as a growth promoter, due to its high energy content (Quitmann *et al.*, 2013). In addition, propylene glycol is often used in dry dog food. Other than its confirmed antibiotic effect (Thomas et al., 1980; Fancher et al., 1996), propylene glycol is acted as a humectant to retain the water content and allowed the dog food

perceived with a moist mouth feeling (Kaplow, 1970; Erickson, 1982). No adverse effect would induce if given the doses under 2000 mg/kg/day to dogs (Mortensen, 1992) and it possessed a very low risk to human body as well (Fowles et al., 2013). Hence, future study can start to examine how to implement the three preservatives (sorbic acid, calcium propionate and propylene glycol) to certain dog food production process to reduce the incident of *Salmonella*.

To develop a more friendly and safe environment between pets and pet owners, some recommendations on preventing *Salmonella* infection from dog food are provided. First, pet owners should try to reduce the possibility of mixing water with dry dog food. Practically, to avoid accidently rehydrating dry dog food, food and drinking water should be fed to dogs in separate areas and pet owners should take extra care to ensuring the dry condition of the feeding bowls and utensils. Second, if dry dog food have to be rehydrated to reach a desired palatable and soft texture, dog food should be fed to dogs and have them finished as soon as possible to keep the pathogen, if any, from growing. Third, dog owners should follow proper hygiene practices, for instance, before and after feeding dogs or having physical contact with dogs.

Chapter 6. Conclusions & Suggestions for Future Research

In this study, 26 brands of different commercial dog food were examined. The 72-hr growth/decline kinetics for a cocktail of three *Salmonella* strains on dog food was obtained. They were rehydrated to a moisture level of 35% and were stored under 30°C. Twenty-two (22) out of 26 dog foods supported the survival or the growth of *Salmonella*. The growth kinetics curves were best fitted with reparameterized Gompertz model. The distributions of the three parameters (N_{max}, μ_{max}, λ) in the fitted reparameterized Gompertz model were collected, which could be applied in future microbial quantitative risk assessment studies. Conversely, four brands of dog food inhibited the growth of *Salmonella* with different inactivation rates. Those decline curves were fitted with log-linear model. The results indicated that the condition where dry dog food was rehydrated to 35% moisture level and was stored under 30°C could be a favorable environment for *Salmonella* to grow. The different behavior of *Salmonella* in rehydrated dry dog food may be attributed to dog food intrinsic factors including pH, a_w, and possible antimicrobial ingredient effect.

In order to obtain more robust results, more parallel repeats on the brands of dog food examined in this study should be performed. Additionally, how *Salmonella* will behave on other dog foods should also be characterized to develop a broader and deeper understanding. Moreover, chemical analysis examinations should be performed to identify and quantify the compositions of dog food, and thus statistical analyses can be conducted to assess the factors affecting the growth of *Salmonella*.

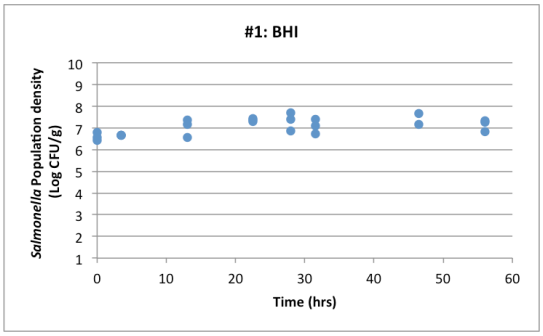
Furthermore, research is also needed to identify and categorize the role of other microorganisms present in dog food. Residential micro flora may compete with

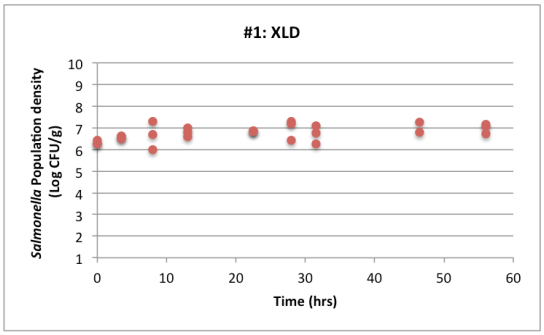
Salmonella for growing space and nutrients. Thus, research should explore if there is any possible interaction between those flora and *Salmonella*, which can help provide insights into developing new prevention and mitigation strategies.

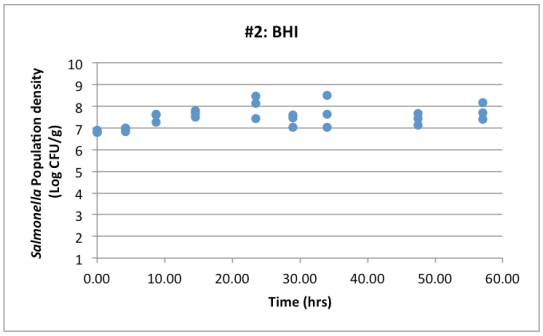
Few studies have been conducted on rehydrated dog food or animal feeds, but the impact of rehydration is not negligible. Thus, a potential rehydration step should be explored in depth. Rehydration should be taken considerations when investigating reported outbreaks. More studies and surveys should be further conducted to understand feeding practices. For instance, it is important to recognize the prevalence of rewetting dog food and the reasons for the practice.

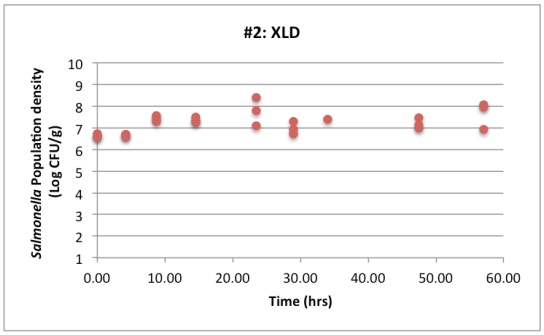
Chapter 7. Appendices

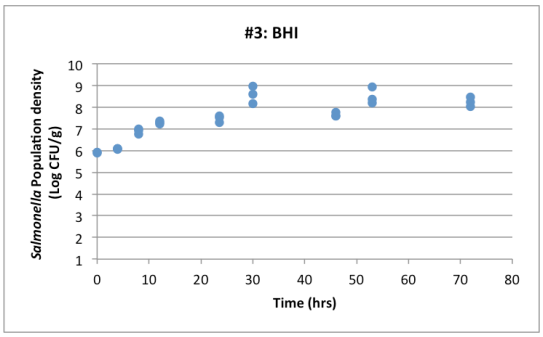
Appendix A: Growth/decline curves for 26 brands of dog food

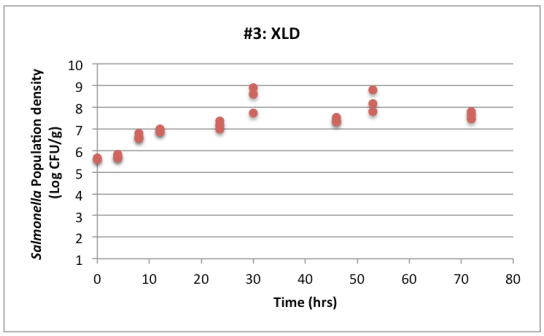


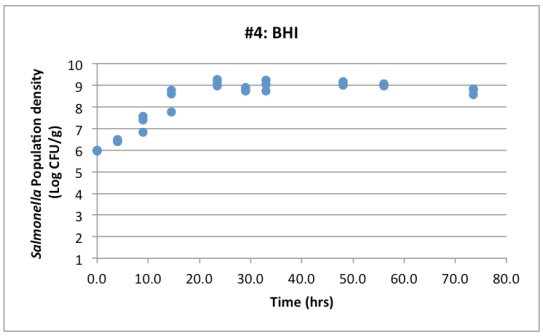


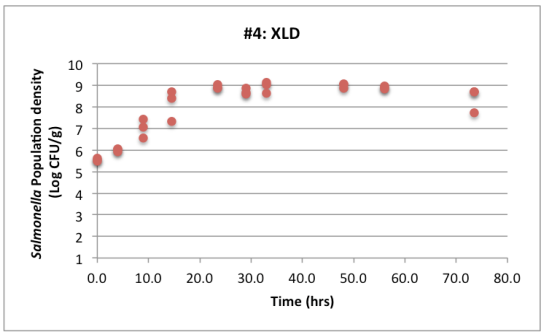


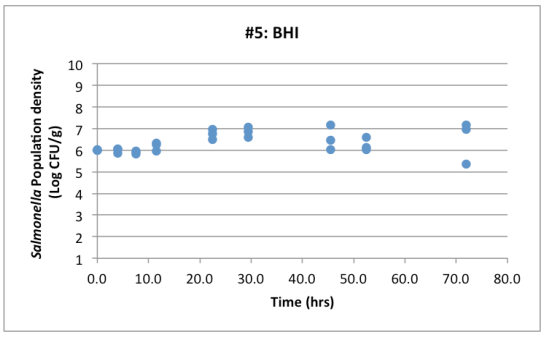


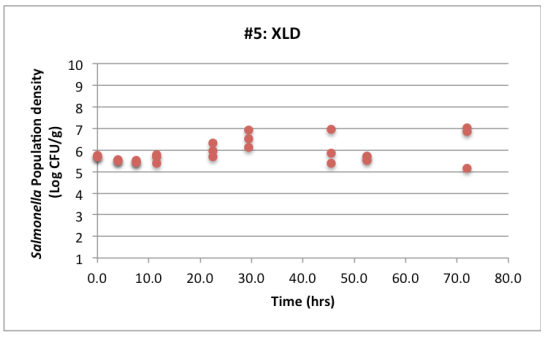


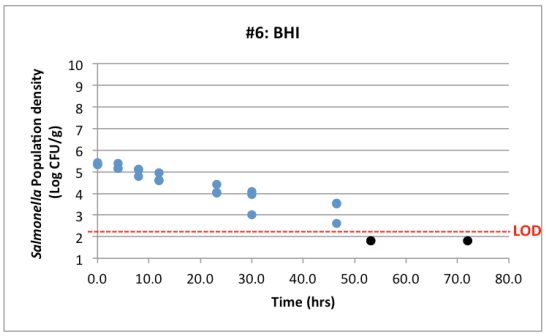


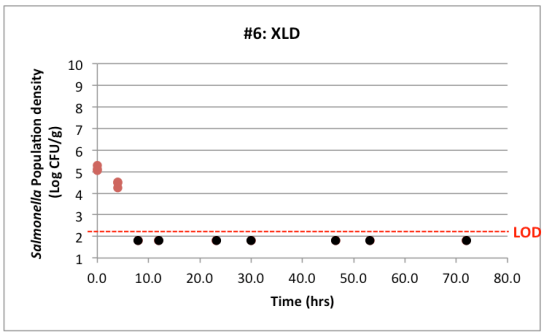


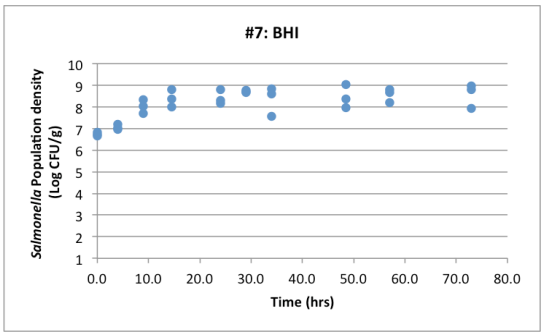


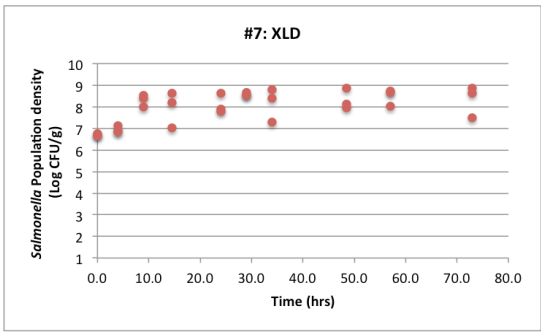

#5: BHI
Salmonella Population density (Log CFU/g)
Time (hrs)

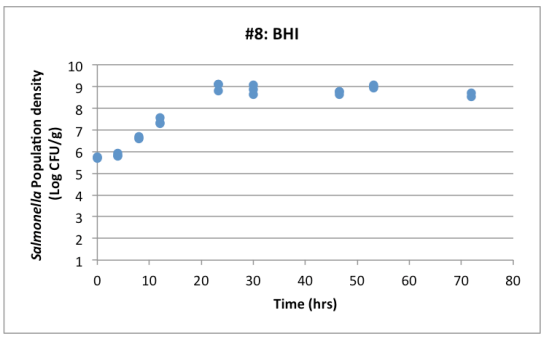

#5: XLD
Salmonella Population density (Log CFU/g)
Time (hrs)

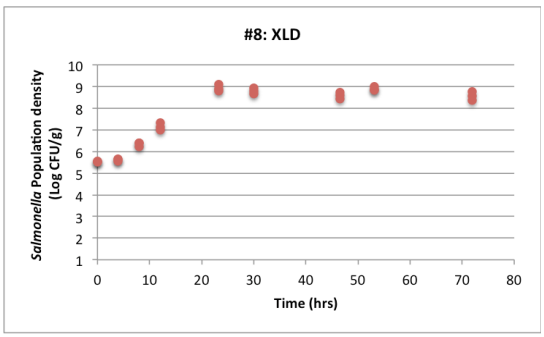

#6: BHI
Salmonella Population density (Log CFU/g)
LOD
Time (hrs)

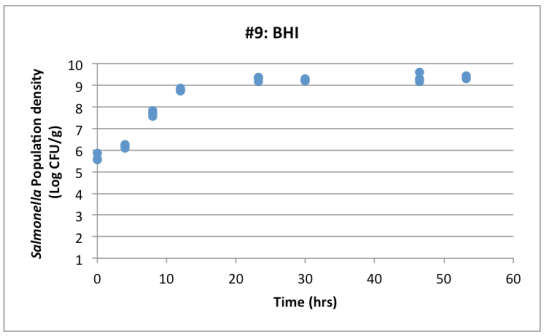

#6: XLD
Salmonella Population density (Log CFU/g)
LOD
Time (hrs)

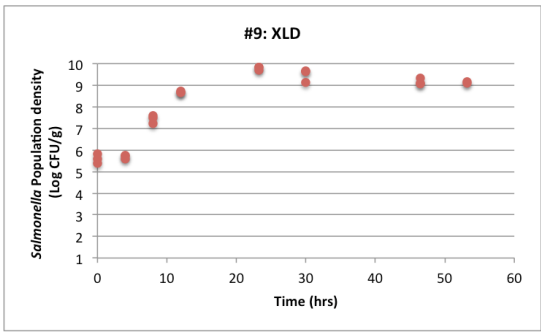

#7: BHI
Salmonella Population density (Log CFU/g)
Time (hrs)

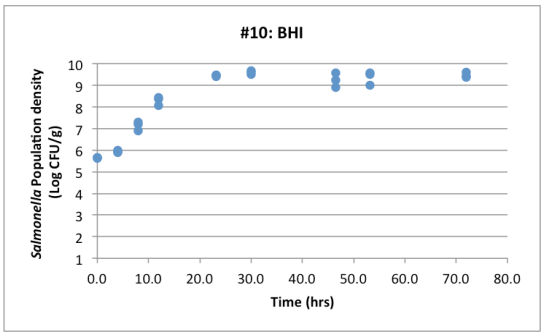

#7: XLD
Salmonella Population density (Log CFU/g)
Time (hrs)

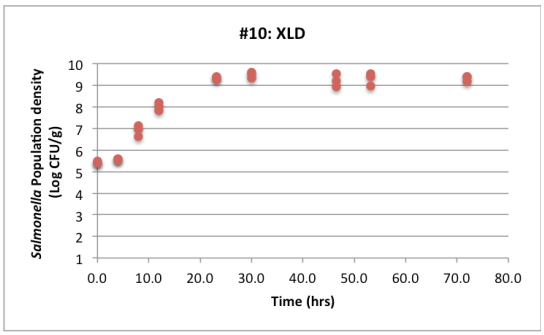

#8: BHI
Salmonella Population density (Log CFU/g)
Time (hrs)

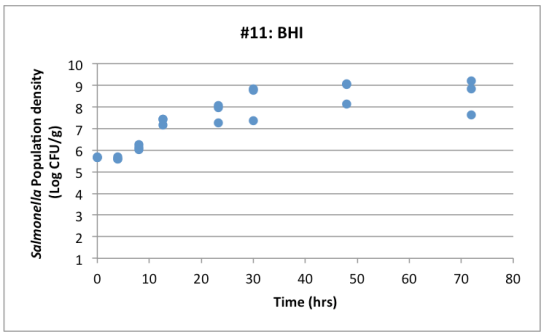

#8: XLD
Salmonella Population density (Log CFU/g)
Time (hrs)

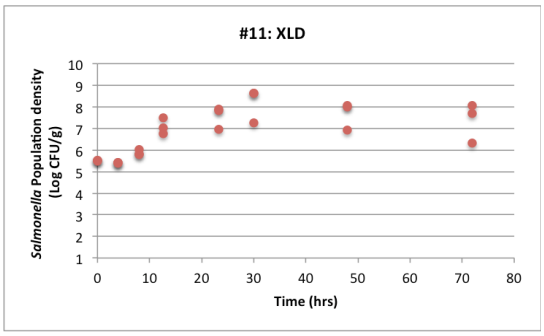

#9: BHI
Salmonella Population density (Log CFU/g)
Time (hrs)

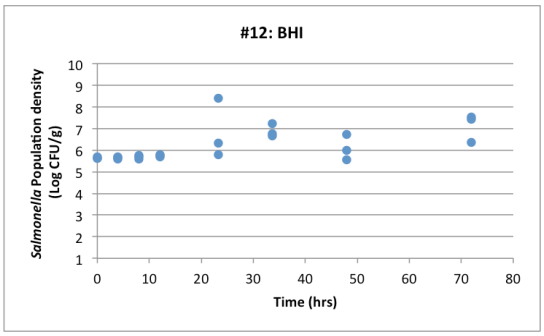

#9: XLD
Salmonella Population density (Log CFU/g)
Time (hrs)


#10: BHI
Salmonella Population density (Log CFU/g)
Time (hrs)


#10: XLD
Salmonella Population density (Log CFU/g)
Time (hrs)


#11: BHI
Salmonella Population density (Log CFU/g)
Time (hrs)


#11: XLD
Salmonella Population density (Log CFU/g)
Time (hrs)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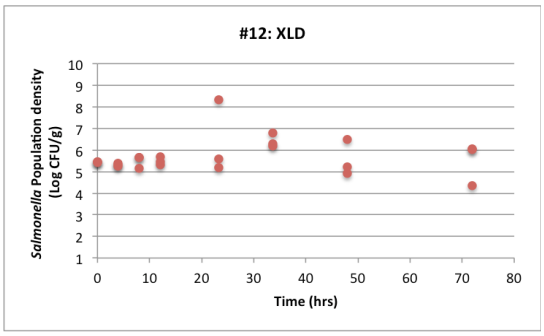
Salmonella Population density (Log CFU/g)
Time (hrs)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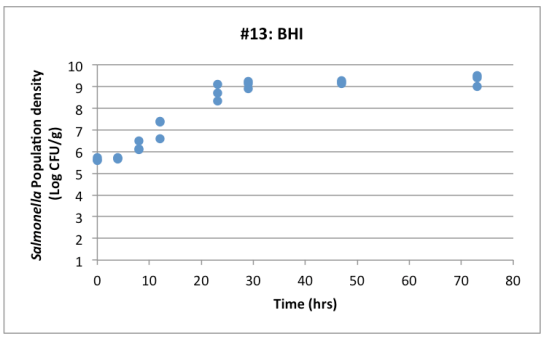
Salmonella Population density (Log CFU/g)
Time (hrs)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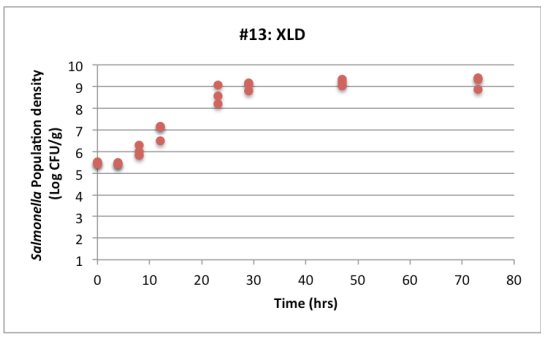
Salmonella Population density (Log CFU/g)
Time (hrs)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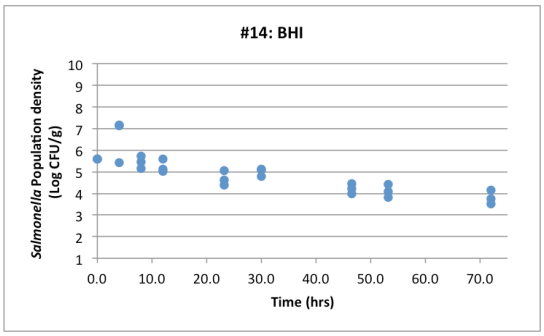
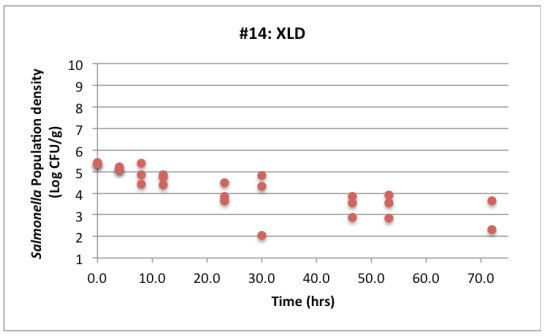
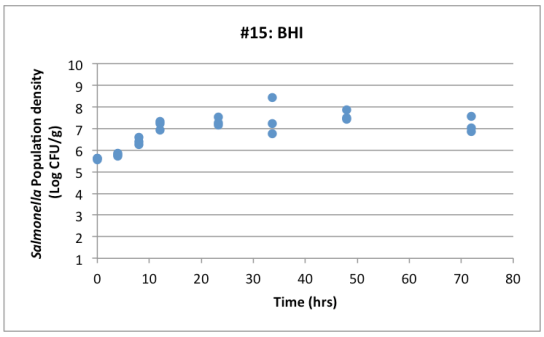
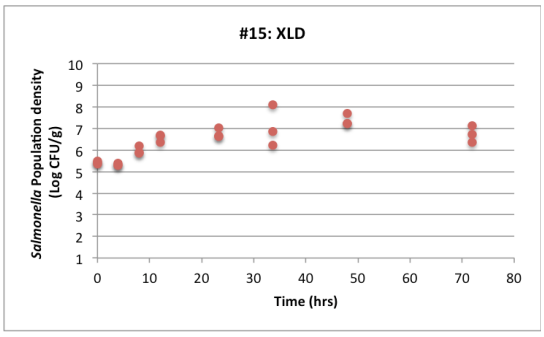
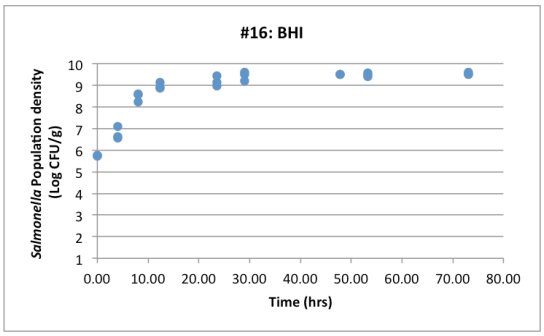
Salmonella Population density (Log CFU/g)
Time (hrs)

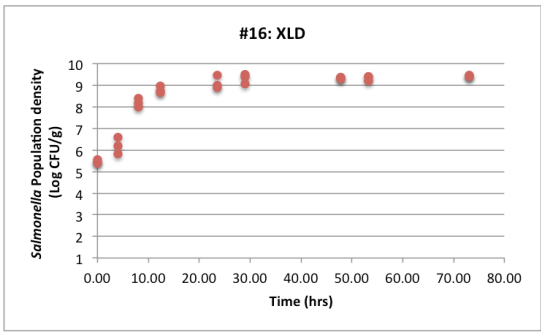

#14: BHI
Salmonella Population density (Log CFU/g)
Time (hrs)

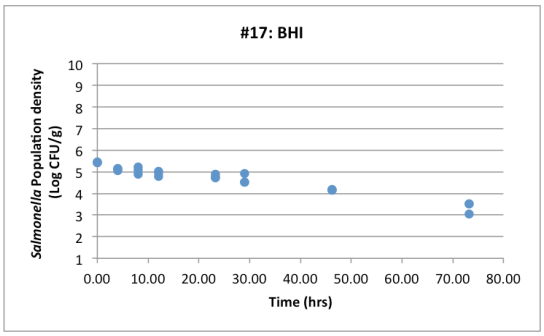

#14: XLD
Salmonella Population density (Log CFU/g)
Time (hrs)


#15: BHI
Salmonella Population density (Log CFU/g)
Time (hrs)


#15: XLD
Salmonella Population density (Log CFU/g)
Time (hrs)


#16: BHI
Salmonella Population density (Log CFU/g)
Time (hrs)


#16: XLD
Salmonella Population density (Log CFU/g)
Time (hrs)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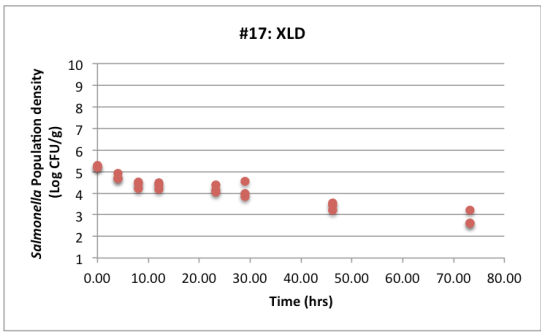
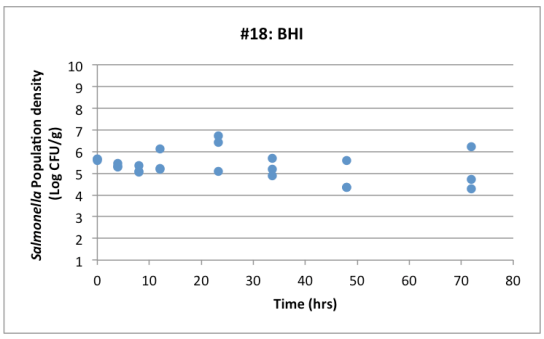
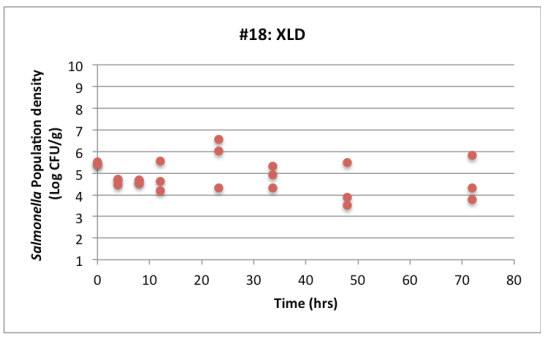
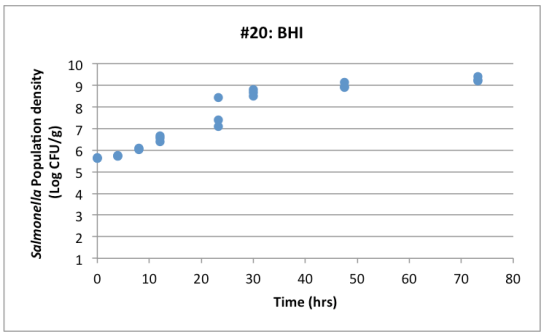
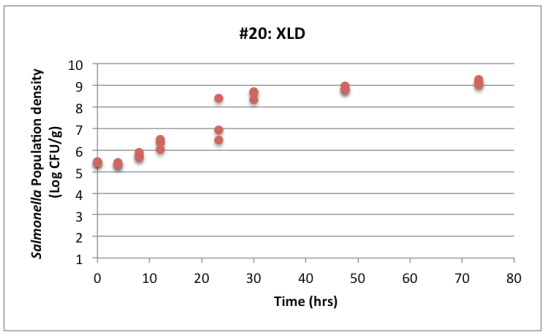
Salmonella Population density (Log CFU/g)
Time (hrs)

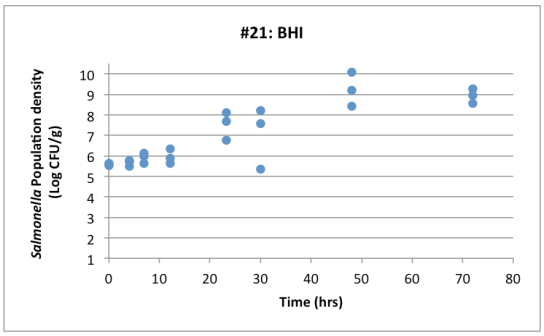

#17: XLD
Salmonella Population density (Log CFU/g)
Time (hrs)

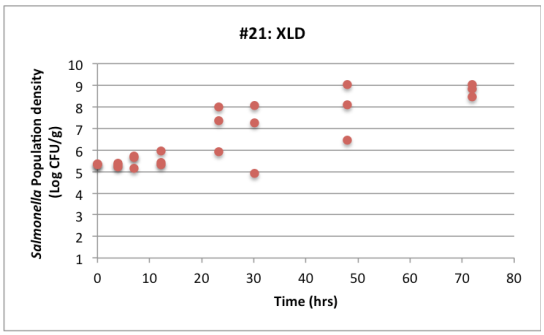

#18: BHI
Salmonella Population density (Log CFU/g)
Time (hrs)

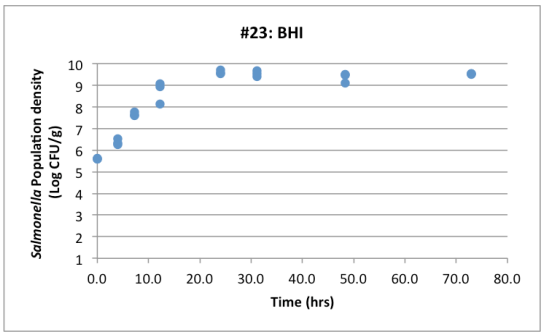

#18: XLD
Salmonella Population density (Log CFU/g)
Time (hrs)

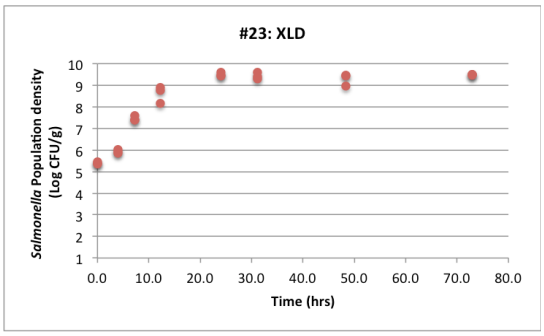

#20: BHI
Salmonella Population density (Log CFU/g)
Time (hrs)

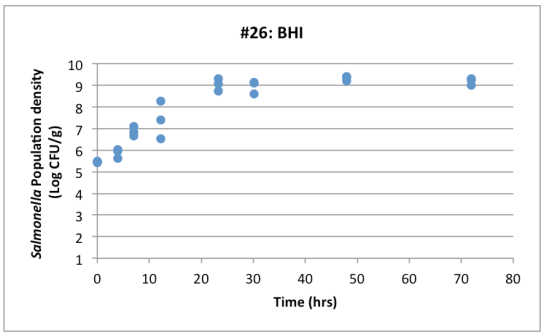

#20: XLD
Salmonella Population density (Log CFU/g)
Time (hrs)

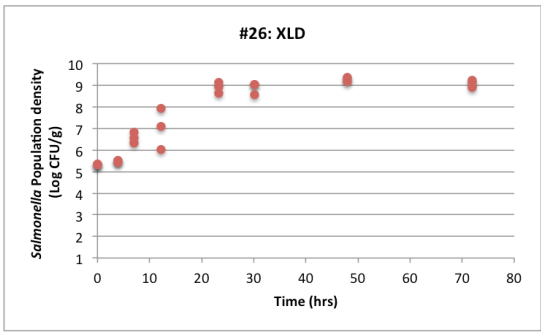

#21: BHI
Salmonella Population density (Log CFU/g)
Time (hrs)


#21: XLD
Salmonella Population density (Log CFU/g)
Time (hrs)


#23: BHI
Salmonella Population density (Log CFU/g)
Time (hrs)


#23: XLD
Salmonella Population density (Log CFU/g)
Time (hrs)


#26: BHI
Salmonella Population density (Log CFU/g)
Time (hrs)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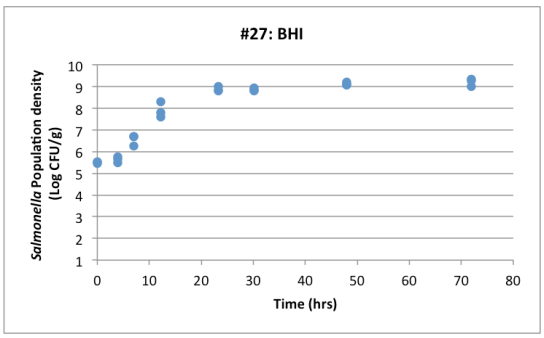
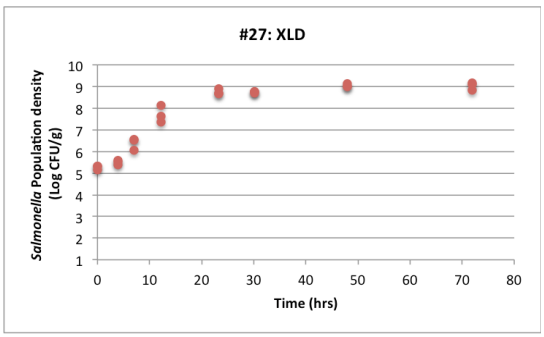
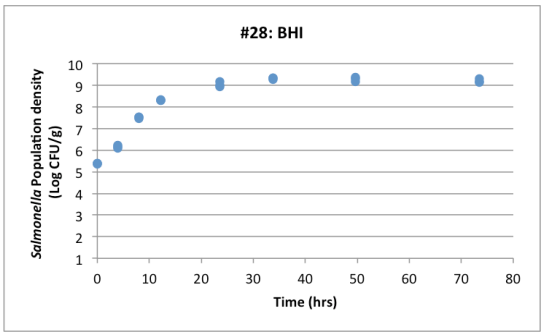
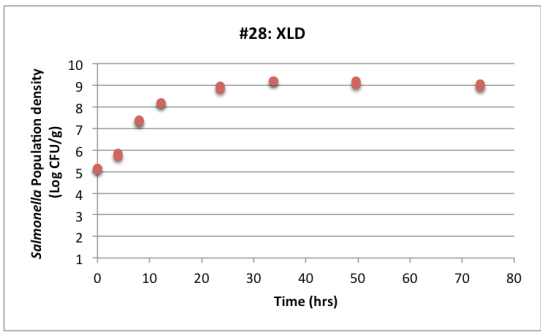
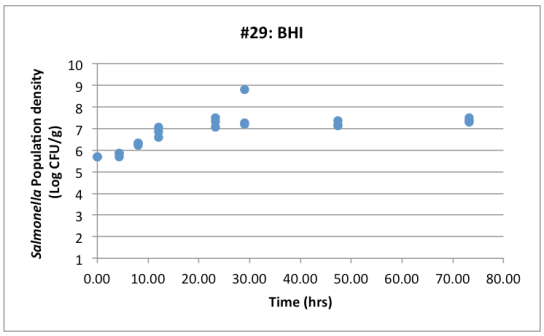
Salmonella Population density (Log CFU/g)
Time (hrs)

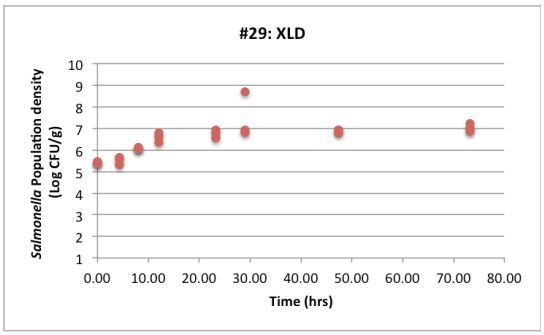

#27: BHI
Salmonella Population density (Log CFU/g)
Time (hrs)

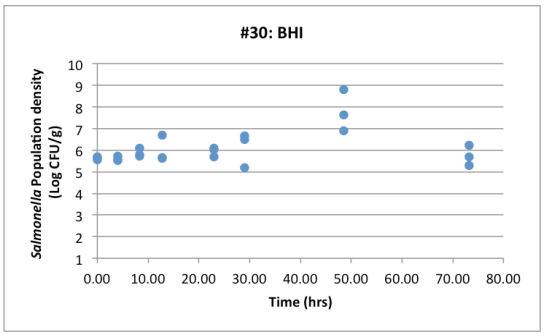

#27: XLD
Salmonella Population density (Log CFU/g)
Time (hrs)

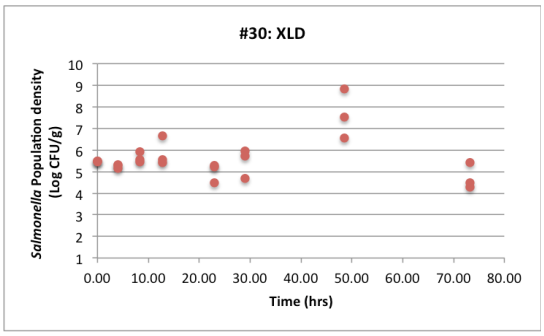

#28: BHI
Salmonella Population density (Log CFU/g)
Time (hrs)


#28: XLD
Salmonella Population density (Log CFU/g)
Time (hrs)

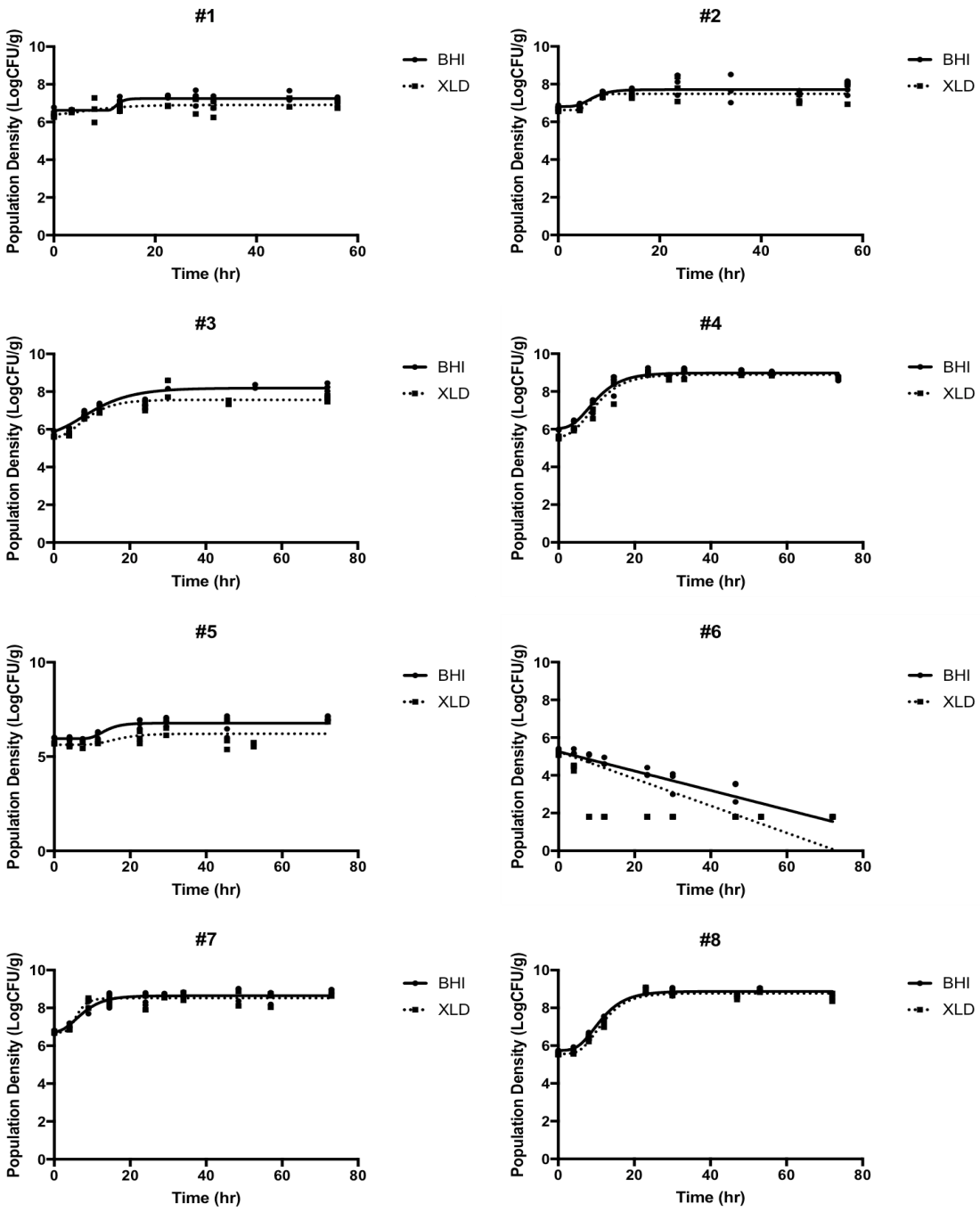

#29: BHI
Salmonella Population density (Log CFU/g)
Time (hrs)

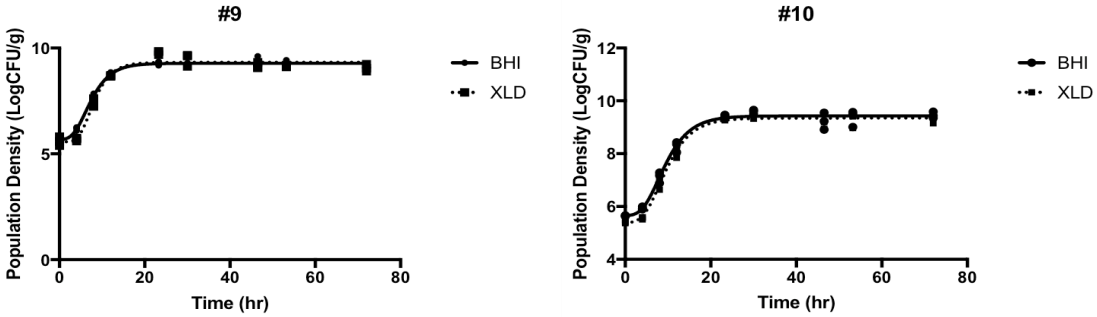

#29: XLD
Salmonella Population density (Log CFU/g)
Time (hrs)


#30: BHI
Salmonella Population density (Log CFU/g)
Time (hrs)

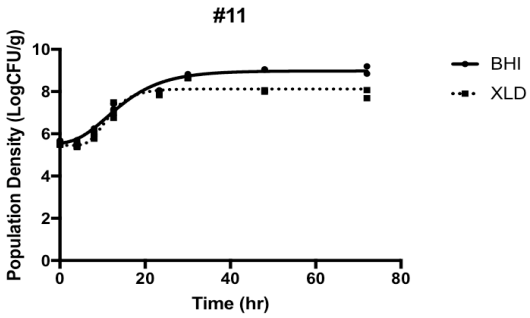

#30: XLD
Salmonella Population density (Log CFU/g)
Time (hrs)

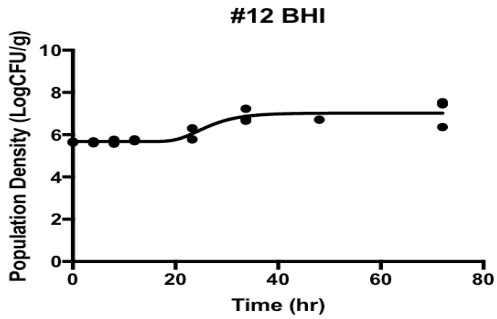
Appendix B: Reparameterized Gompertz models and log-linear models fitted for 26 brands of dog food

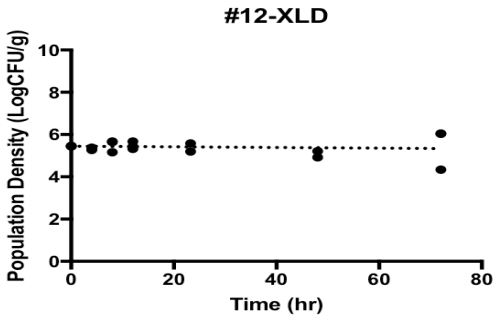


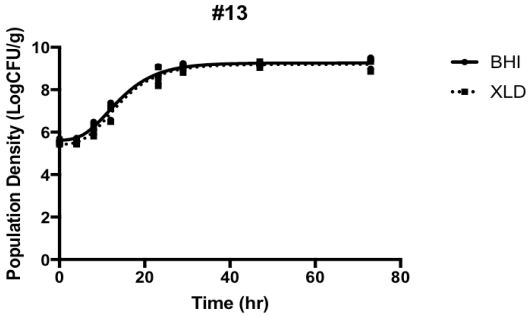

#9
Population Density (LogCFU/g)
Time (hr)
BHI
XLD

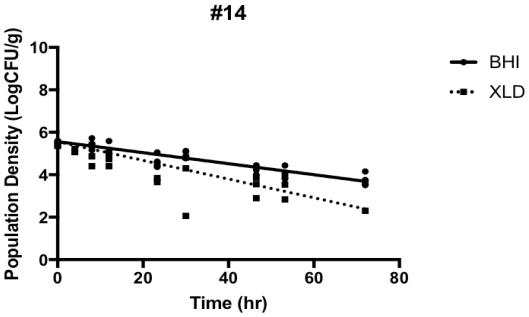
#10
Population Density (LogCFU/g)
Time (hr)
BHI
XLD

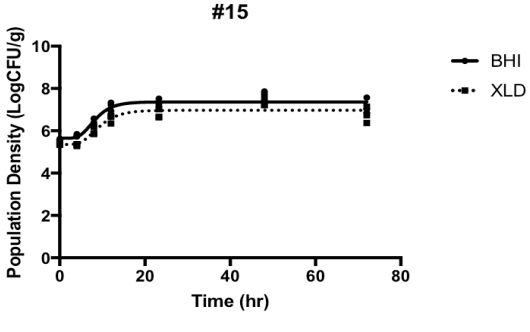

#11
Population Density (LogCFU/g)
Time (hr)
BHI
XLD

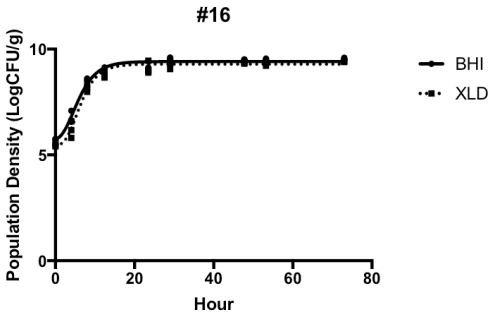

#12 BHI
Population Density (LogCFU/g)
Time (hr)

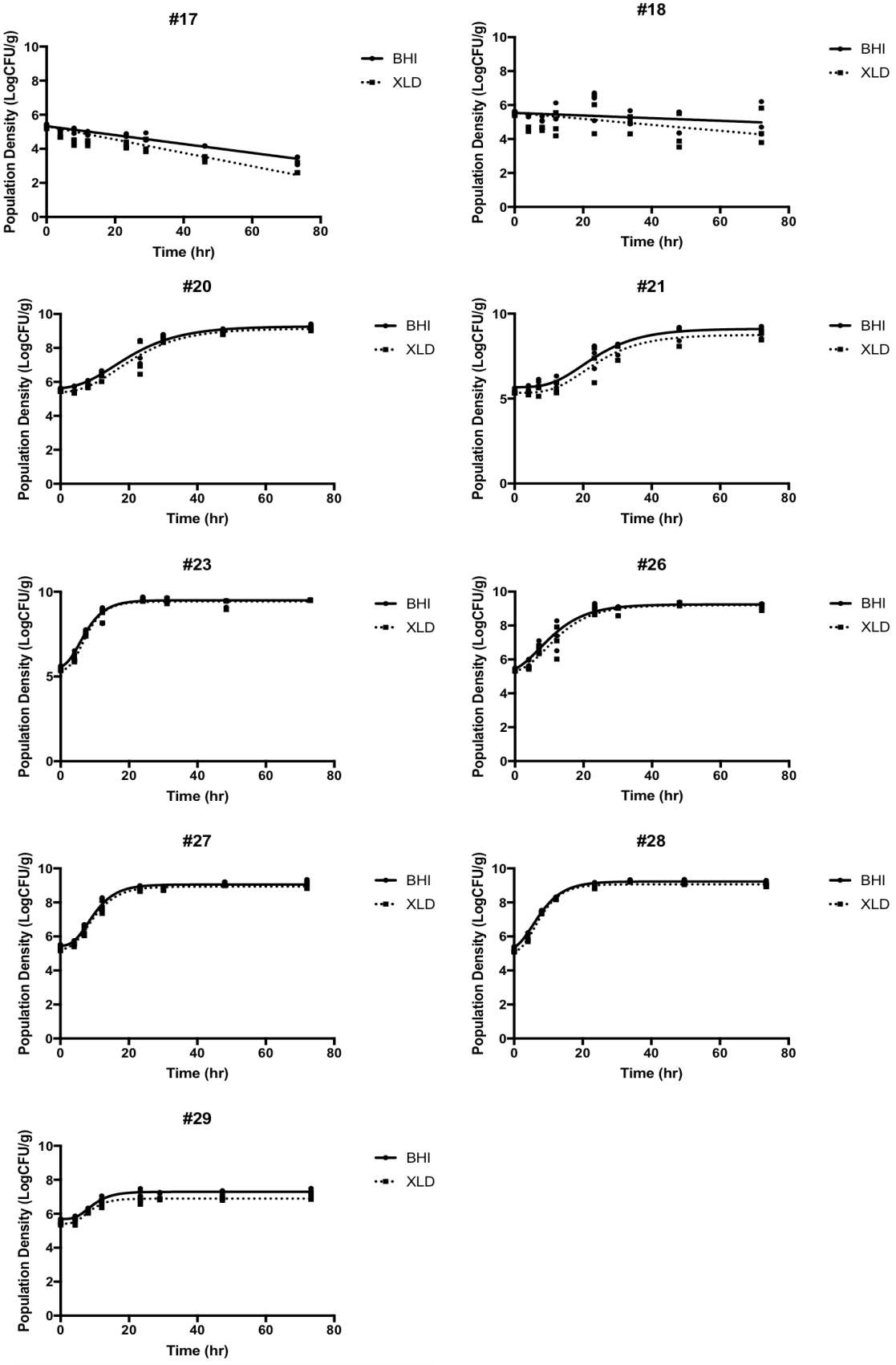

#12-XLD
Population Density (LogCFU/g)
Time (hr)

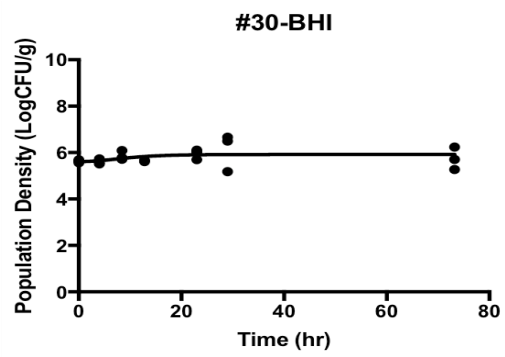

#13
Population Density (LogCFU/g)
Time (hr)
BHI
XLD

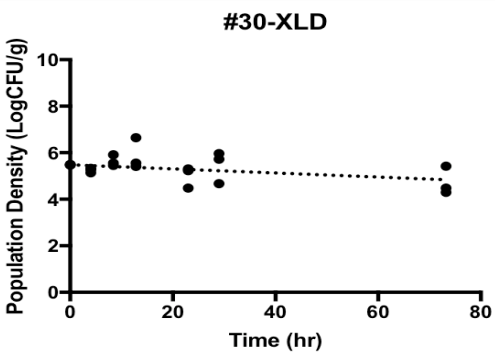

#14
Population Density (LogCFU/g)
Time (hr)
BHI
XLD


#15
Population Density (LogCFU/g)
Time (hr)
BHI
XLD


#16
Population Density (LogCFU/g)
Hour
BHI
XLD


#17
#18
#20
#21
#23
#26
#27
#28
#29
Population Density (LogCFU/g)
Time (hr)
BHI
XLD


#30-BHI
Population Density (LogCFU/g)
10
8
6
4
2
0
0
20
40
60
80
Time (hr)


#30-XLD
Population Density (LogCFU/g)
10
8
6
4
2
0
0
20
40
60
80
Time (hr)

Chapter 8. References

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