FINAL REPORT
September 1, 2014

Correlation of Water Activity to Survival, Recovery of Injured & Growth of Pathogenic Salmonella in Rendered Animal Products under Different Temperature and Relative Humidity Storage Conditions

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Date Submitted: September 1, 2014

Project Start Date: July 1, 2013

Duration of Project: 12 months

Lay Summary:
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Water activity describes the available water in food that can support the growth of bacteria, yeasts and molds. Water activity is defined as the vapor pressure of water in the substance divided by the vapor pressure of pure water at the same temperature.

Water activity and moisture content are not the same measurement. Often, it is assumed that foods with higher moisture content will have higher water activity levels than dry foods. However, this is not always correct. It is possible to have foods with the same moisture content but very different water activities due to a variety of different reasons. Because of this misnomer, dry foods are often thought to be safe, shelf-stable products (Podolak et. al, 2010). Yet, these products can still provide marginal survival and growth conditions for an infection-causing dose of bacteria (Podolak et. al, 2010; FDA, 2012).

FDA reports that the minimum reported a_w for *Salmonella* spp is 0.94 (FDA 2013). However, *Salmonella* species have the ability grow at water activity levels low as 0.91 (Dilbaghi and Sharma 2007) and some researchers suggest the lowest water activity for *Salmonella* growth may be lower. Long-term survival of *Salmonella* (greater than 8 month) has been reported in foods with water activities as low as 0.18 (Kotzekidou, 1998). Beuchat (2009) reported that *Salmonella* can adapt to extreme environmental conditions and survival is enhanced at low water activity. Beuchat further reported that thermal tolerance is enhanced at pH near 7 and at low water activity.

Christian and Scott (1953) discovered that water activity (A_w) is the only measurement of water in foods that correlates with *Salmonella* growth. Although Salmonella growth will decrease due osmotic shock at low water activity levels, *Salmonella* can persist and survive in dry or low moisture environments for years depending on the food product (Mossel et al, 1965; Tysset and Durand, 1976). The survivability of *Salmonella* in the low-moisture, finished rendered animal feeds is of great importance to the rendering industry, particularly due to the FDA's zero tolerance regulation for pet foods. The purpose of this project was examine the relationship between water activity and moisture content in finished rendered meals and to determine if a cocktail of *Salmonella* Enteritidis, *Salmonella* Newport, *Salmonella* Choleraesuis, and *Salmonella* Dublin (four of the eight pathogenic strains identified by the FDA as hazardous in animal feeds) can survive and grow in storage in different humidity levels in these low moisture, commercial products.

The results of this study supported the findings of previous research conducted in foods. Initially, the rendered meal samples were inoculated with a *Salmonella* cocktail and incubated for 14 days without added humidity. In the poultry and beef meals, the *Salmonella* population declined due to osmotic shock and low available water; however, at day 14, *Salmonella* was still detected. In the second part of this study, the uninoculated, rendered meal samples were exposed to 50, 70, and 90% humidity levels at 28°C (83°F), and thus, the water activity levels of each sample in each humidity level increased gradually over 96 hours. The water activity levels increased to a range in which *Salmonella* species can readily survive and potentially grow. Additionally, an experiment was conducted indicating that the *Salmonella* population decreased after inoculation of 7 logarithms ($\log_{10}$) in the rendered meals stored at 90% humidity at 28°C

(83°F). Although the *Salmonella* levels decreased on average of 2-3 $\log_{10}$ cfu/g in the meals, *Salmonella* survived and was still detectable after 24, 48, and 72 hours of storage. Finally, the rendered meal samples were adjusted to a_w of approximately 0.7, 0.8 and 0.9 and inoculated with 9 $\log_{10}$ of a *Salmonella* cocktail. During this experiment, the *Salmonella* population declined after during the first 24 hour after inoculation. Despite the higher adjusted water activity levels in some of the samples, the *Salmonella* did not recover during the 96 hour incubation period.

Objective (s):

a) Using commercially available rendered meal samples, examine the relationship between water activity and moisture content.

b) Using commercially available rendered meal samples, determine the water activity and survivability of a cocktail containing *Salmonella* Choleraesuis, *Salmonella* Enteriditis, *Salmonella* Newport and *Salmonella* Dublin over various storage times at 28°C (83°F) with no added humidity

c) By adjusting the water activities of commercially available rendered meal samples, determine the water activity level and recoverability of a cocktail containing *Salmonella* Choleraesuis, *Salmonella* Enteriditis, *Salmonella* Newport and *Salmonella* Dublin over various storage times at 28°C (83°F) with no added humidity.

d) Determine the water activity and survivability of a cocktail containing *Salmonella* Choleraesuis, *Salmonella* Enteriditis, *Salmonella* Newport and *Salmonella* Dublin over various storage times at 50, 70, and 90% humidity levels at 28°C (83°F) in commercially available rendered meal samples,

Project Overview:

Introduction:

Water activity or a_w is the ratio of partial vapor pressure of water in a substance divided by the standard state partial vapor pressure of water. It is the unit that shows how much available water in the food matrix microorganisms can utilize (Barbosa-Canovas et al 2008). The minimum reported a_w for *Salmonella* spp is 0.94 (FDA 2013). In 1969, Liu et al. showed that when the moisture level of meat and bone meal is increased, the heat resistant of *Salmonella* decreases, with this declining effect starting between 15% to 20% moisture levels. Also, artificially inoculated *Salmonella* Seftenberg had lower heat resistance than naturally contaminated *Salmonella*. In 2005, Carlson et al. investigated the relationship between water activity and *Salmonella* survival rate in ground turkey. The data showed that the rate of thermal inactivation of *Salmonella* cocktail (8 strains) decreased 64% when increase the ground turkey water activity from 0.95 to 0.99 at 60ºC, this data supports that low water activity can increase *Salmonella* thermal resistance.

The pathogenicity of many bacterial strains can be increased depending on the osmo-regulatory capacities, composition of food and processing environments. In this study, the data indicated osmotic shock can decreased *Salmonella* populations in the rendered animal meals. Under certain conditions, if pathogenic bacteria are present in high numbers in dry food, or the infective dose is low, or the products rehydrate during manufacture processing or storage environment,

then the risk to consumer remains.

Water Activity Experiments

Determination of Water Activity and Moisture Content Measurement

Background

Salmonella spp. are Gram-negative, rod shaped pathogenic bacteria that can originate from a variety of sources and cause contamination problems for both the food and rendered animal feed industries. Dry and/or low moisture products including animal feed, flour, spices, chocolate, powders, herbs are often considered to be very stable, safe, long shelf life products. These products are usually high in proteins, carbohydrates and other nutrients, which are the essential nutrients for microorganisms including pathogenic bacteria. Animal feed, spices and food products have been associated with *Salmonella* contamination. In 1962, Burr and Helmboldt examined 436 animal by-product samples and reported 12.8% were positive for 10 different serotypes of *Salmonella*. In 1963, Isa et al. reported 43 out of 281 animal feed and feed constituent samples were *Salmonella* positive (about 15%).

Data indicates *Salmonella* can persist for long periods of time in a wide range of dried food products. This study was designed to determine survival of *Salmonella* in low water activity rendered animal products. Water activity or a_w is the ratio of partial vapor pressure of water in a substance divided by the standard state partial vapor pressure of water. It is the unit that shows how much available water is in the food matrix that microorganisms can utilize (Barbosa-Canovas et al 2008). The minimum reported a_w for *Salmonella* spp is 0.94 (FDA 2013). However, after the outbreaks noted in peanut butter, the minimum recommended a_w for dry foods or ingredients is being questioned. There has been limited research conducted regarding the survival rate of pathogenic *Salmonella* serotypes in rendered animal meals and its mechanism for survival in such low water activity environment.

Purpose:

Water activity levels and moisture contents of pet-food grade and feed-grade poultry and beef meat and bone meals as well as feather meal and blood meals were measured to determine if there was a correlation between the two different types of measurements. Also, this experiment would determine if the water activity levels of the meals were within a survivable range for *Salmonella* species.

Materials and Methods:

Rendering Meal Sample Collection

Samples of pet-food grade and feed-grade poultry and beef meat and bone meals as well as feather meal and blood meal were received from rendering plants in the U.S. All samples were stored at room temperature in sealed, 1-gallon, Ziploc® bags until needed for experimentation.

Water Activity and Moisture Content

The initial water activity of each sample was measured in duplicate in water activity sample cups (40107, Decagon Devices, Inc, Pullman, WA) using a dew-point water activity meter (Aqualab

series 3TE, Decagon Devices, Inc., Pullman, WA). Initial moisture content in each meal sample was determined in duplicate by three separate drying methods as follows: infrared radiation drying using an infrared moisture analyzer (IR-35, Denver Instrument, Denver, CO, USA), forced convection oven (FD-53, Binder Inc., Bohemia, NY) drying at 135 C for 2 h (AOAC 930.15), and forced convection oven (FD-53, Binder Inc., Bohemia, NY) drying at 104 C for 3 h (Thiex and Van Erem, 1999).

To determine moisture content via the infrared moisture analyzer (IR-35, Denver Instrument, Denver, CO, USA), 11.0 x 2.9 cm aluminum drying pans (25433-022, VWR Scientific Products, Suwanee, GA) containing approximately 2-3 g of each meal sample were placed into the instrument. The hood of the instrument was then lowered to automatically signal the beginning of the moisture analysis test. Moisture content (%) results for each meal sample were reported on the instrument's screen.

To ascertain moisture content via the oven drying methods, a 5.7 x 1.6 cm aluminum drying pan with tab (25433-008, VWR Scientific Products, Suwanee, GA) was weighed (NewClassic ML4002E/03, Mettler Toledo International, Inc., Greifensee, Switzerland) and the tared weight (T) was recorded to the nearest 0.01 g. Each aluminum drying pan was tared to zero while on the balance and then 2 g of each well-mixed meal sample was added each tared pan. The weight of each meal sample was recorded to the nearest 0.01g (M). Each meal sample was evenly distributed in the aluminum drying pan by gently shaking and then placed into a preheated oven (FD-53, Binder Inc., Bohemia, NY).

The aluminum drying pans containing approximately 2 g of each meal sample were dried using each of the two oven drying methods: forced air oven drying at 135°C for 2 h (AOAC 930.15) and forced air oven drying at 104°C for 3 h (Thiex and Van Erem, 1999). Then aluminum drying pans containing the samples were allowed to cool in a dessicator. Once the dried samples cooled, the weight of the pan and dried samples was measured and recorded (S). The moisture content (%) of each sample was determined by

Moisture Content (%) = 100-[(S-T)*100/M]

Results:

No correlations were apparent between moisture content measurements and water activity levels of the meals samples. The three moisture analysis methods produced very variable results.

Samples did have water activity levels that were within the survivable range of *Salmonella*.

Table 1. Average water activity and percentage moisture measured by three methods in rendered animal products (± standard deviation).

Sample Code #	Sample	Average Water Activity Level	Standard Deviation	Average Moisture Content at 135C for 2 h (%)	Standard Deviation (%)	Average Moisture Content at 104C for 3h (%)	Standard Deviation (%)	Average Moisture Content from Moisture Analyzer (IR 35)(%)	Standard Deviation (%)
2A/B	Poultry By-Product Meal	0.3435	0.001	6.75	2.121	5.75	3.536	7.08	0.346
3A/B	Poultry By-Product Meal	0.276	0.003	2.50	1.414	2.00	2.828	3.26	0.177
4A/B	Poultry By-Product Meal	0.2805	0.011	1.50	0.000	2.25	2.121	6.09	3.627
5A/B	Poultry By-Product Meal	0.341	0.133	2.75	0.707	3.50	1.414	3.17	0.226
6A/B	Poultry By-Product Meal	0.326	0.000	5.00	1.414	3.75	2.121	5.40	0.276
7A/B	Poultry By-Product Meal	0.299	0.003	3.25	0.707	2.50	1.414	3.46	0.049
8A/B	Poultry By-Product Meal	0.31	0.001	3.75	3.536	2.25	0.707	3.92	0.255
14A/B	Poultry By-Product Meal	0.313	0.010	1.75	0.707	3.25	2.121	3.47	0.297
20A/B	Poultry By-Product Meal	0.2945	0.016	2.00	0.000	1.75	0.707	2.98	0.962
21A/B	Poultry By-Product Meal	0.3415	0.074	2.75	0.707	2.50	1.414	3.31	0.007
22A/B	Poultry By-Product Meal	0.246	0.001	3.00	2.828	2.00	1.414	3.84	0.438
23A/B	Poultry By-Product Meal	0.3205	0.001	2.50	1.414	1.50	0.000	3.27	0.170
24A/B	Poultry By-Product Meal	0.3235	0.037	2.75	2.121	8.50	18.385	3.51	0.078
25A/B	Poultry By-Product Meal	0.281	0.034	2.50	1.414	1.50	0.000	2.86	0.375
26A/B	Poultry By-Product Meal	0.316	0.023	1.25	2.121	1.25	0.707	3.20	0.156
27A/B	Poultry By-Product Meal	0.316	0.041	4.50	1.414	8.25	13.435	5.09	0.205
28A/B	Poultry By-Product Meal	0.3725	0.028	28.00	4.243	4.75	0.707	5.40	0.191
29A/B	Poultry By-Product Meal	0.337	0.001	5.00	1.414	4.25	0.707	5.55	0.085
30A/B	Poultry By-Product Meal	0.3265	0.006	3.75	2.121	3.50	2.828	4.84	0.453
31A/B	Poultry By-Product Meal	0.309	0.010	3.50	0.000	2.75	0.707	4.50	0.276
38A/B	Medium Ash Poultry Meal	0.3465	0.042	2.75	2.121	2.25	0.707	3.69	0.163
40A/B	Medium Ash Poultry Meal	0.2995	0.018	3.00	0.000	3.50	1.414	4.79	0.396
42A/B	Poultry Meal	0.2545	0.002	3.00	2.828	1.50	0.000	3.58	0.283

45A/B	Poultry Meal	0.2905	0.011	3.00	1.414	0.75	0.707	3.55	0.304
46A/B	Medium Ash Poultry Meal	0.2455	0.006	2.50	0.000	1.75	2.121	3.78	0.255
47A/B	Poultry Meal	0.356	0.000	4.50	2.828	3.00	2.828	3.33	0.255
48A/B	Poultry Meal	0.278	0.008	2.50	2.828	2.25	0.707	3.40	0.156
51A/B	Medium Ash Poultry Meal	0.2785	0.008	4.00	0.000	1.25	0.707	4.27	0.127
57A/B	Poultry Meal	0.3325	0.013	4.00	0.000	4.50	1.414	3.69	1.563
60A/B	Poultry Meal	0.2885	0.011	3.00	2.828	1.75	0.707	4.02	0.042
64A/B	Medium Ash Poultry Meal	0.248	0.007	1.50	7.071	2.50	2.828	4.87	0.672
65A/B	Medium Ash Poultry Meal	0.501	0.042	11.25	0.707	5.75	16.263	9.81	0.750
66A/B	Poultry Meal	0.2525	0.005	2.00	4.243	4.00	2.828	3.90	0.057
71A/B	Medium Ash Poultry Meal	0.296	0.031	3.75	4.950	2.50	0.000	4.43	0.071
72A/B	Medium Ash Poultry Meal	0.3315	0.071	3.00	2.828	3.50	1.414	5.04	0.184
75A/B	Poultry Meal	0.288	0.010	1.25	0.707	1.50	5.657	2.78	0.262
76A/B	Medium Ash Poultry Meal	0.233	0.044	2.75	3.536	0.75	2.121	3.68	0.078
77A/B	Poultry Meal	0.2995	0.002	3.75	2.121	0.75	0.707	3.64	0.304
80A/B	Poultry Meal	0.3515	0.056	4.75	0.707	2.50	1.414	4.39	0.035
82A/B	Medium Ash Poultry Meal	0.3375	0.008	2.25	2.121	2.50	0.000	3.62	0.049
86A/B	Poultry By-Product Meal	0.2295	0.053	1.00	1.414	2.25	2.121	2.03	0.000
89A/B	Poultry By-Product Meal	0.204	0.030	1.00	1.414	3.75	6.364	1.88	0.481
90A/B	Poultry By-Product Meal	0.239	0.048	1.50	1.414	0.50	0.000	2.34	0.226
98A/B	Poultry By-Product Meal	0.2335	0.018	3.75	2.121	2.75	0.707	3.86	0.064
116A/B	Poultry By-Product Meal	0.283	0.001	2.00	0.000	2.50	2.828	3.33	0.361

Table 1 (continued). Average water activity and percentage moisture measured by three methods in rendered animal products (± standard deviation).

Sample Code #	Sample	Average Water Activity Level	Standard Deviation	Average Moisture Content at 135C for 2 h (%)	Standard Deviation (%)	Average Moisture Content at 104C for 3h (%)	Standard Deviation (%)	Average Moisture Content from Moisture Analyzer (IR 35)(%)	Standard Deviation (%)
9A/B	Meat and Bone Meal	0.4285	0.008	7.25	0.354	5.25	4.950	7.00	0.361
10A/B	Meat and Bone Meal	0.3495	0.030	8.50	0.707	8.25	0.707	7.61	0.276
11A/B	Meat and Bone Meal	0.3985	0.008	6.25	0.354	5.00	2.828	6.18	0.318
12A/B	Meat and Bone Meal	0.334	0.001	7.00	0.707	6.25	0.707	6.78	0.389
13A/B	Meat and Bone Meal	0.405	0.008	6.50	0.707	4.50	5.657	6.45	0.304
17A/B	Blended MBM	0.299	0.008	4.25	2.475	2.75	2.121	3.49	0.318
18A/B	Blended MBM	0.327	0.017	4.00	1.414	3.75	0.707	4.25	0.474
36A/B	Beef and Pork Meal	0.2545	0.016	2.50	0.000	3.50	0.000	3.81	0.184
41A/B	Beef and Pork Meal	0.338	0.001	3.00	0.000	1.25	3.536	3.77	0.106
52A/B	Beef and Pork Meal	0.305	0.042	4.50	1.061	3.25	0.707	4.48	0.233
54A/B	Beef and Pork Meal	0.241	0.003	2.75	0.707	1.25	0.707	3.03	0.290
59A/B	Beef and Pork Meal	0.2215	0.009	2.50	0.707	1.25	0.707	2.72	0.170
62A/B	Beef and Pork Meal	0.286	0.001	3.50	1.061	2.25	2.121	3.92	0.212
73A/B	Beef and Pork Meal	0.266	0.016	1.75	0.000	1.75	2.121	4.20	0.523
74A/B	Beef and Pork Meal	0.2795	0.019	3.50	0.354	1.75	0.707	4.26	0.354
79A/B	Beef and Pork Meal	0.319	0.010	3.25	2.828	3.75	4.950	3.79	0.049
85A/B	Beef and Pork Meal	0.294	0.020	1.50	0.354	2.25	2.121	3.35	0.247
87A/B	Meat and Bone Meal	0.365	0.030	5.25	1.061	3.00	0.000	4.62	0.198
88A/B	Blended MBM	0.375	0.055	4.75	0.707	1.50	7.071	4.54	0.085
96A/B	Meat and Bone Meal	0.474	0.065	6.00	0.707	6.50	0.000	6.67	0.417
100A/B	Meat and Bone Meal	0.36	0.004	7.75	0.354	7.00	2.828	6.72	0.148
101A/B	Meat and Bone Meal	0.465	0.018	5.75	0.354	6.75	2.121	6.23	0.502
114A/B	Meat and Bone Meal	0.298	0.004	6.00	0.707	7.00	5.657	5.90	0.191
115A/B	Meat and Bone Meal	0.3355	0.008	4.75	1.768	4.75	0.707	5.03	0.099

Table 1 (continued). Average water activity and percentage moisture measured by three methods in rendered animal products (± standard deviation).

Sample Code #	Sample	Average Water Activity Level	Standard Deviation	Average Moisture Content at 135C for 2 h (%)	Standard Deviation (%)	Average Moisture Content at 104C for 3h (%)	Standard Deviation (%)	Average Moisture Content from Moisture Analyzer (IR 35)(%)	Standard Deviation (%)
15A/B	Feather Meal	0.355	0.020	6.25	0.354	5.50	4.243	7.18	0.792
16A/B	Feather Meal	0.331	0.008	7.25	0.354	4.25	3.536	7.04	0.283
32A/B	Feather Meal	0.235	0.001	4.75	1.768	2.25	0.707	4.13	0.184
33A/B	Feather Meal	0.255	0.001	3.25	0.354	2.75	6.364	4.40	0.368
34A/B	Feather Meal	0.368	0.000	4.25	1.061	4.25	0.707	4.63	0.184
35A/B	Feather Meal	0.3185	0.012	3.00	0.000	3.50	2.828	4.55	0.092
39A/B	Feather Meal	0.518	0.000	7.75	0.354	5.00	2.828	7.42	0.035
44A/B	Feather Meal	0.464	0.115	9.75	1.061	8.50	0.000	9.56	0.049
49A/B	Feather Meal	0.5325	0.026	12.00	0.000	10.75	2.121	11.29	0.028
55A/B	Feather Meal	0.4655	0.025	8.50	0.707	8.25	2.121	8.26	0.092
58A/B	Feather Meal	0.318	0.061	3.50	0.000	2.75	0.707	4.15	0.141
63A/B	Feather Meal	0.535	0.030	11.50	1.414	16.75	10.607	12.61	0.467
67A/B	Feather Meal	0.45	0.000	8.00	1.414	6.75	0.707	7.28	0.120
69A/B	Feather Meal	0.345	0.000	8.75	1.061	7.25	0.707	7.61	0.834
78A/B	Feather Meal	0.3515	0.005	31.00	4.243	6.50	2.828	9.01	0.304
81A/B	Feather Meal	0.444	0.003	10.00	0.000	9.75	0.707	10.07	0.078
92A/B	Feather Meal	0.3385	0.005	7.25	0.354	6.25	2.121	6.97	0.035
97A/B	Feather Meal	0.3315	0.001	3.50	0.000	2.75	3.536	4.39	0.014
99A/B	Feather Meal	0.3065	0.004	4.25	0.354	1.75	4.950	4.54	0.156
110A/B	Feather Meal	0.3	0.057	4.75	1.061	3.75	0.707	4.80	0.170
111A/B	Feather Meal	0.324	0.011	7.50	1.414	4.75	0.707	5.49	0.382
112A/B	Feather Meal	0.353	0.017	5.25	0.354	3.75	0.707	5.42	0.113
113A/B	Feather Meal	0.3105	0.006	3.50	0.707	3.75	3.536	3.65	0.106

Table 1 (continued). Average water activity and percentage moisture measured by three methods in rendered animal products (± standard deviation).

Sample Code #	Sample	Average Water Activity Level	Standard Deviation	Average Moisture Content at 135C for 2 h (%)	Standard Deviation (%)	Average Moisture Content at 104C for 3h (%)	Standard Deviation (%)	Average Moisture Content from Moisture Analyzer (IR 35)(%)	Standard Deviation (%)
19A/B	Blood Meal	0.579	0.052	11.00	0.000	10.00	2.828	10.40	0.665
37A/B	Blood Meal	0.428	0.001	8.75	0.354	7.50	0.000	9.11	0.057
43A/B	Blood Meal	0.381	0.008	9.50	0.707	8.25	2.121	9.81	0.156
50A/B	Blood Meal	0.5715	0.001	9.25	1.061	11.25	9.192	9.31	0.283
53A/B	Blood Meal	0.5135	0.002	9.75	0.354	9.00	1.414	9.57	0.255
56A/B	Blood Meal	0.577	0.001	9.00	0.707	9.00	1.414	9.05	0.014
61A/B	Blood Meal	0.5675	0.030	8.00	1.414	8.00	1.414	8.58	0.092
68A/B	Blood Meal	0.239	0.024	8.75	0.354	9.75	2.121	8.72	0.368
70A/B	Blood Meal	0.5175	0.001	8.00	0.707	9.75	2.121	8.39	0.240
83A/B	Blood Meal	0.362	0.006	9.25	0.354	9.50	0.000	7.86	2.277
84A/B	Blood Meal	0.442	0.001	8.75	1.061	9.25	4.950	9.39	0.156
91A/B	Blood Meal	0.655	0.004	14.75	0.354	14.25	2.121	8.46	6.329
93A/B	Blood Meal	0.515	0.031	8.00	0.707	8.50	1.414	8.44	0.219
94A/B	Blood Meal	0.593	0.098	14.00	0.707	14.50	1.414	11.50	1.478
95A/B	Blood Meal	0.375	0.010	11.25	1.061	9.00	0.000	9.38	0.007

Table 1 (continued). Average water activity and percentage moisture measured by three methods in rendered animal products (± standard deviation).

Sample Code #	Sample	Average Water Activity Level	Standard Deviation	Average Moisture Content at 135C for 2 h (%)	Standard Deviation (%)	Average Moisture Content at 104C for 3h (%)	Standard Deviation (%)	Average Moisture Content from Moisture Analyzer (IR 35)(%)	Standard Deviation (%)
102A/B	Plant #1*	0.3425	0.018	2.75	2.475	6.25	7.778	3.66	1.237
103A/B	Plant #2*	0.3635	0.002	4.00	0.707	4.00	0.000	4.27	0.191
104A/B	Plant #3*	0.3185	0.004	3.25	0.354	3.00	0.000	4.45	0.453
105A/B	Plant #4*	0.361	0.000	4.50	0.707	3.50	0.000	4.66	0.064
106A/B	Plant #5*	0.318	0.001	4.50	0.000	2.75	3.536	3.76	2.065
107A/B	Plant #6*	0.2965	0.012	3.75	1.061	3.50	2.828	4.69	0.219
108A/B	Plant #7*	0.259	0.001	2.00	0.707	2.00	1.414	3.84	0.219
109A/B	Plant #8*	0.4365	0.084	7.25	1.061	7.00	4.243	7.87	0.537

*Renderer did not identify product

High Humidity Storage and Impact on Water Activity

Introduction:

The water activity of pet-food grade and feed-grade poultry and beef meat and bone meals was measured after 24, 48 and 96 h time intervals of storage in 50%, 70% and 90% humidity levels. The purpose of the study was to determine if the meal samples absorbed moisture and if water activity levels were impacted. No *Salmonella* was added to the meals for this experiment.

Materials and Methods:

Rendering Meal Sample Collection

Samples of pet-food grade and feed-grade poultry and beef meat and bone meals were supplied by rendering plants in the U.S. Twelve poultry by-product meal samples and twelve beef meal samples were randomly selected for this experiment. All samples were stored at room temperature in sealed, 1-gallon, Ziploc® bags until needed for experimentation.

Water Activity

The initial water activity of each sample was measured in duplicate in water activity sample cups (40107, Decagon Devices, Inc, Pullman, WA) using a dew-point water activity meter (Aqualab series 3TE, Decagon Devices, Inc., Pullman, WA).

Environmental Chamber

An environmental chamber was retrofitted to allow temperature and humidity modifications. Temperature and humidity of the environmental chamber unit were monitored by a thermometer and humidity meter (90113-1, Springfield Precision, Oak Brook, IL). The humidity was controlled via a 1-gallon humidifier (EE5301O, Crane USA, Bensenville, IL) connected to an electronic thermohygrostat controller with a humidity sensor (Plug and Play, Thermomart, Toronto, Canada). This unit allowed the humidity to be precisely controlled and monitored within the environmental chamber.

Humidity Trials

Samples of poultry and beef meat and bone meals used for testing were randomly selected prior to experimentation. Each meat and bone meal sample was weighed (10 g) in duplicate into large 11x 2.9 cm aluminum pans (25433-022, VWR Scientific Products, Suwanee, GA). Each uncovered sample was placed in the environmental chamber (702-ASHR4, Labline Environette, Labline Instruments, Inc., Melrose Park, IL) set at 50% humidity at 28°C (83°F) for 24 h. Additional samples of the same products were stored under the same conditions for 48 h and 96 h. The experiment was repeated at each 70% and 90% humidity levels at 28°C (83°F). The water activity of each of the samples was measured after the appropriate time interval. The initial water activity was re-measured in two randomly selected meal samples prior to humidity exposure to determine if water activity changed while the meal samples were stored in 1-gallon, Ziploc® bags. All samples upon removal from the environmental chamber were immediately sealed in 1-gallon, Ziploc® bags until water activity measurements. For water activity measurement, samples were vigorously shaken and then transferred in duplicate (approximately 2-3 g) into water activity cups. The samples then were sealed in the water activity cups with parafilm (52858-032,

VWR Scientific Products, Suwanee, GA and stored at room temperature in sealed, 1.5-gallon, Rubbermaid® storage container until water activity was measured and recorded (within 2 to 4 hours of sample collection).

Results:

Water activity levels of each poultry and beef meal sample increased with exposure to 50, 70, and 90% humidity levels at 28°C (83°F) when subsamples were measured at 24, 48, and 96 h (Figures 9-14). The poultry rendered meals were grouped together according to their initial water activity levels. The low initial water activity group had levels ranging from 0.204-0.239. The medium initial water activity level group had levels ranging from 0.246-0.268. The high initial water activity level group had levels ranging from 0.3515-0.501. After exposure to 50% humidity levels at 28°C (83°F) for 96 h, the low initial water activity group increased to an average water activity level of 0.552±0.006. The medium initial water activity level group had reached an average water activity level of 0.557±0.007. The high initial water activity level group increased its average water activity level to 0.560±0.015 (Figure 1).

The beef rendered meals were grouped together according to their initial water activity levels. The low initial water activity group had levels ranging from 0.2215-0.286. The medium initial water activity level group had levels ranging from 0.327-0.3405. The high initial water activity level group had levels ranging from 0.4285-0.474. Exposure to 50% humidity levels at 28°C (83°F) for 96 h, the low initial water activity group increased to an average water activity level of 0.558±0.009. The medium initial water activity level group had reached an average water activity level of 0.529±0.005. The high initial water activity level group increased its average water activity level to 0.571±0.004 (Figure 2).

After exposure to 70% humidity levels at 28°C (83°F) for 96 h, the low initial water activity group for poultry meals increased to an average water activity level of 0.737±0.017. The medium initial water activity level group had reached an average water activity level of 0.730±0.017. The high initial water activity level group increased its average water activity level to 0.714±0.026 (Figure 3).

After exposure to 70% humidity levels at 28°C (83°F) for 96 h, the low initial water activity group for beef meals rose to an average water activity level of 0.745±0.012. The medium initial water activity level group had reached an average water activity level of 0.734±0.005. The high initial water activity level group increased its average water activity level to 0.735±0.003 (Figure 5).

After exposure to 90% humidity levels at 28°C (83°F) for 96 h, the low initial water activity group for poultry meals increased to an average water activity level of 0.976±0.004. The medium initial water activity level group had reached an average water activity level of 0.979±0.005. The high initial water activity level group increased its average water activity level to 0.988±0.010 (Figure 3). The water activity levels increased to a range in which *Salmonella* species can survive and potential grow. The minimum reported a_w for the growth of *Salmonella* species is 0.94 (FDA 2013).

Exposure to 90% humidity levels at 28°C (83°F) for 96 h led to the low initial water activity group for beef meals increasing to an average water activity level of 0.968±0.006. The medium initial water activity level group increased to an average water activity level of 0.963±0.002. The high initial water activity level group reached an average water activity level to 0.966±0.004 (Figure 6). Similar to the poultry meal products, the water activity levels gradually increased with exposure to humidity to a range in which *Salmonella* species can persist and multiply. The minimum reported a_w for the growth of *Salmonella* species is 0.94 (FDA 2013).

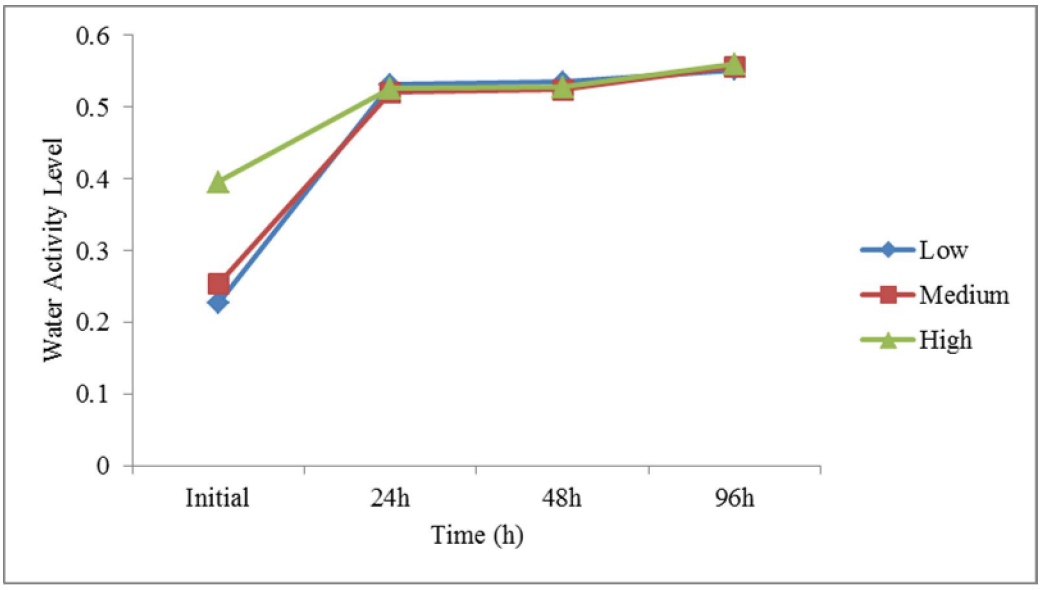

Figure 1. Water activity levels in randomly selected, poultry rendered meal samples prior to humidity exposure as well as 24, 48, and 72 h at 50% humidity at 28°C (83°F).[1]

[1]To calculate averages and to show overall trends, the poultry rendered meals were grouped together according to their initial water activity levels. The low initial water activity group had levels ranging from 0.204-0.239. The medium initial water activity level group had levels ranging from 0.246-0.268. The high initial water activity level group had levels ranging from 0.3515-0.501.

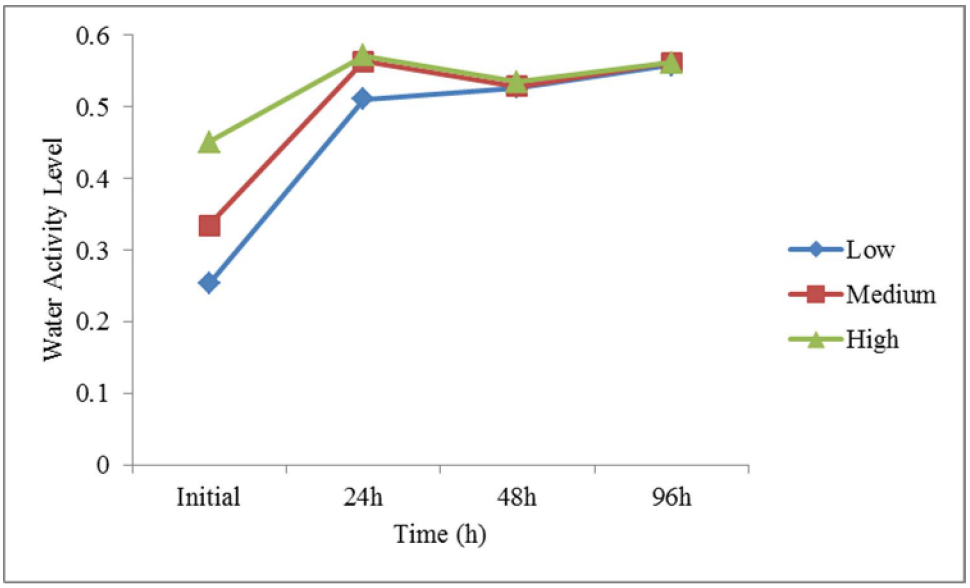

Figure 2. Water activity levels in randomly selected, beef rendered meal samples prior to humidity exposure as well as 24, 48, and 72 h at 50% humidity at 28°C (83°F). [1]

[1]To calculate averages and to show overall trends, the beef rendered meals were grouped together according to their initial water activity levels. The low initial water activity group had levels ranging from 0.2215-0.286. The medium initial water activity level group had levels ranging from 0.327-0.3405. The high initial water activity level group had levels ranging from 0.4285-0.474.

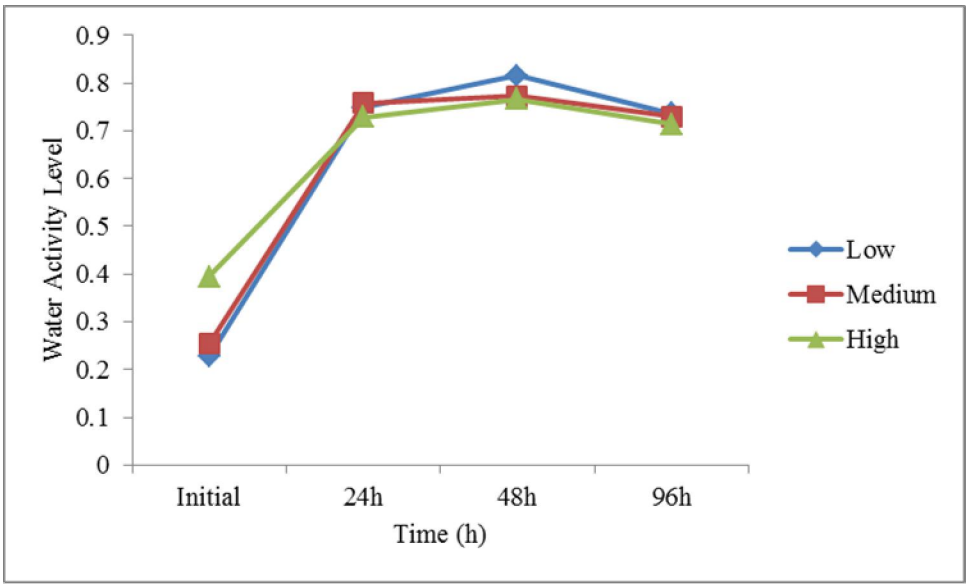

Figure 3. Water activity levels in randomly selected, poultry rendered meal samples prior to humidity exposure as well as 24, 48, and 72 h at 70% humidity at 28°C (83°F). [1]

[1]To calculate averages and to show overall trends, the poultry rendered meals were grouped together according to their initial water activity levels. The low initial water activity group had levels ranging from 0.204-0.239. The medium initial water activity level group had levels ranging from 0.246-0.268. The high initial water activity level group had levels ranging from 0.3515-0.501.

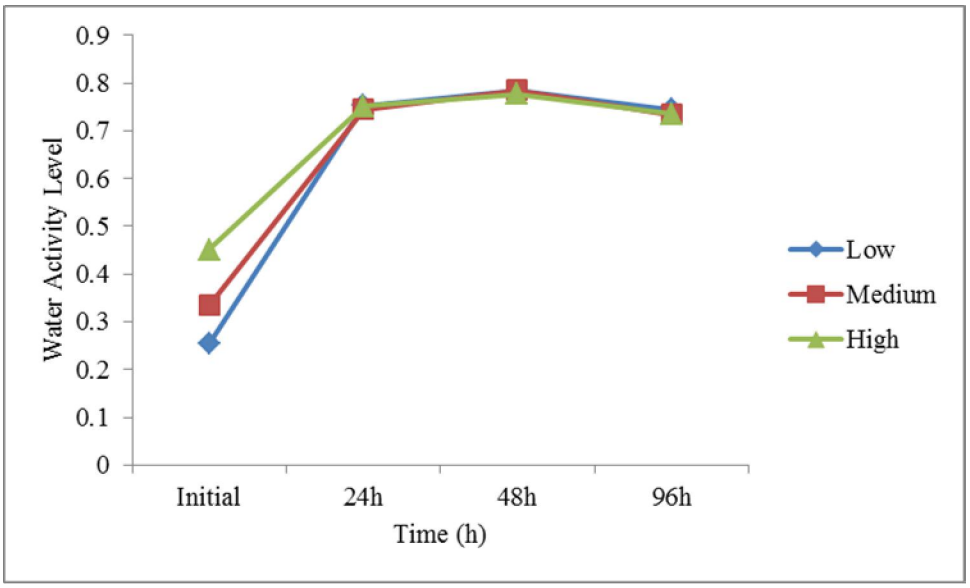

Figure 4. Water activity levels in randomly selected, beef rendered meal samples prior to humidity exposure as well as 24, 48, and 72 h at 70% humidity at 28°C (83°F).[1]

[1]To calculate averages and to show overall trends, the beef rendered meals were grouped together according to their initial water activity levels. The low initial water activity group had levels ranging from 0.2215-0.286. The medium initial water activity level group had levels ranging from 0.327-0.3405. The high initial water activity level group had levels ranging from 0.4285-0.474.

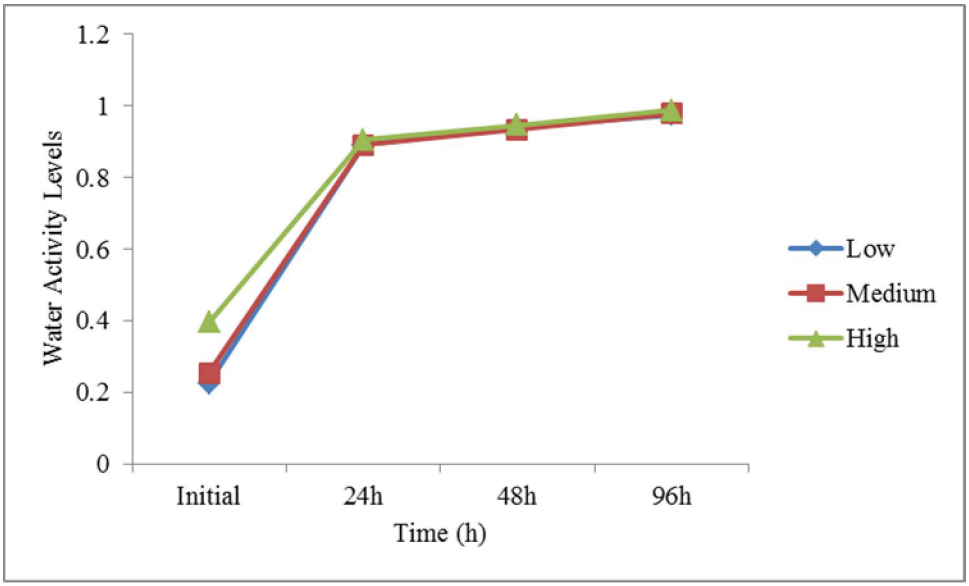

Figure 5. Water activity levels in randomly selected, poultry rendered meal samples prior to humidity exposure as well as 24, 48, and 72 h at 90% humidity at 28°C (83°F). [1]

[1]To calculate averages and to show overall trends, the poultry rendered meals were grouped together according to their initial water activity levels. The low initial water activity group had levels ranging from 0.204-0.239. The medium initial water activity level group had levels ranging from 0.246-0.268. The high initial water activity level group had levels ranging from 0.3515-0.501.

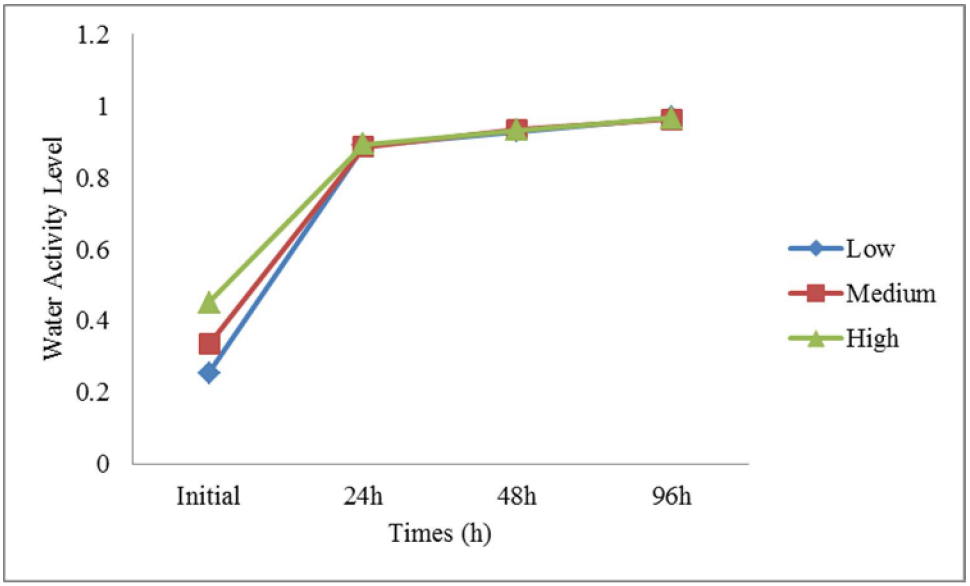

Figure 6. Water activity levels in randomly selected, beef rendered meal samples prior to humidity exposure as well as 24, 48, and 72 h at 90% humidity at 28°C (83°F). [1]

[1]To calculate averages and to show overall trends, the beef rendered meals were grouped together according to their initial water activity levels. The low initial water activity group had levels ranging from 0.2215-0.286. The medium initial water activity level group had levels ranging from 0.327-0.3405. The high initial water activity level group had levels ranging from 0.4285-0.474.

Conclusions:

Exposure of rendered products to high humidity can lead to rapid increases in water activity of the products and can cause the products to develop favorable water activity conditions for microbial growth.

Introduction:

The water activity of pet-food grade and feed-grade poultry and beef meat and bone meals increases when exposed to high humidity levels. Therefore, this experiment was conducted to determine if four of the eight pathogenic strains of *Salmonella* recognized by FDA can grow and/or survive in humid storage conditions in these commercial products.

Materials and Methods:

Rendering Meal Sample Collection

Samples of pet-food grade and feed-grade poultry and beef meat and bone meals were collected from rendering plants in the midwestern, southeastern, and western U.S. Four poultry by-product meal samples and four beef meal samples were randomly selected for this experiment. All samples were stored at room temperature in sealed, 1-gallon, Ziploc® bags until needed for experimentation.

Salmonella Preparation

Salmonella Enteritidis (USDA H4386 serotype, recognized by FDA as hazardous for animal feeds, was obtained for this study from Dr. Vijay Jejuna of the USDA Agricultural Research Service, Microbial Food Safety Research Unit, 600 East Mermaid Lane, Room 2129, Wyndmoor, PA 19038 (FDA, 2010; FDA, 2013). *Salmonella* Enteritidis was grown in 5L of TSB (90000-050, VWR Scientific Products, Suwanee, GA) with 0.1% (wt/vol) yeast extract (0210330390, MP Biomedicals, LLC, Solon, OH) overnight and then concentrated by centrifugation. Centrifugation was conducted at 7,000 x g for 7 min (GSA rotor, DuPont RC5C Sorvall Instruments Centrifuge, DuPont Company, Newtown, CT) at 4°C in sterile centrifuge bottles (47735-696, VWR Scientific Products, Suwanee, GA) and the supernatant was discarded after autoclaving. The pellet was resuspended in 5 mL sterile TSB (90000-050, VWR Scientific Products, Suwanee, GA). The slurry of *Salmonella* Enteritidis was inoculated into each irradiated poultry and beef rendering materials at the rate of 200 µL culture per 20 g sample.

Environmental Chamber Conditions

Temperature and humidity of the environmental chamber unit were monitored by a thermometer and humidity meter (90113-1, Springfield Precision, Oak Brook, IL). The humidity was controlled via a 1-gallon, drop humidifier (EE5301O, Crane USA, Bensenville, IL) connected to an electronic thermohygrostat controller with a humidity sensor (Plug and Play, Thermomart, Toronto, Canada). This unit allowed the humidity to be precisely controlled and monitored within the environmental chamber.

Humidity Trials

Samples of poultry and beef meat and bone meals used for testing were randomly selected prior to experimentation. Each meat and bone meal sample was weighed (20 g) in duplicate into sterile, 50 mL Falcon tubes 89039-660, VWR Scientific Products, Suwanee, GA). The initial water activity was re-measured in two randomly selected meal samples prior to humidity exposure to determine if water activity changed while the meal samples were stored in 1-gallon,

Ziploc® bags. Each sample was inoculated with 200 µL of the *Salmonella* cocktail. Uninoculated samples were included.

As a preliminary experiment to determine how to best distribute the culture within the samples, two hundred µL of crystal violet dye (90008-894, VWR Scientific Products, Suwanee, GA) was added to 20 g each of poultry and beef meal sample in a sterile Falcon tube bag. Crystal violet dye (90008-894, VWR Scientific Products, Suwanee, GA) was used to represent the culture. The mixtures were vortexed (Super Mixer, 1290, Labline Instruments, Inc., Melrose Park, IL) for 2 min on the high setting. Subsamples were observed for color uniformity using a microscope (Meiji MT52104, Meiji Techno Co., Ltd., Santa Clara, CA) at 10x and 40x magnification. Results indicated that a 1:10 ratio of culture to sample and vortex mixing would allow for even distribution of culture throughout each of the poultry and beef meal samples.

Prior to experimentation, a preliminary experiment was conducted to determine if a sterile 0.45µm filter (HVLP002500, EMD Millipore, Bedford, MA) affixed to the top of the open tube with a rubber band (20791966, OfficeMax Incorporated, Naperville, IL) and laboratory tape (89098-062, VWR Scientific Products, Suwanee, GA) would allow moisture to enter the sample tubes. Six sterile 50 mL tubes were filled with 20 g of Indicating DrierRite® (22890-900, VWR Scientific Products, Suwanee, GA). Two of the tubes were topped with the 0.45µm filters, two of the tubes were topped with the loosely fitting caps, and two of the tubes were left opened. All of the tubes were placed into the environmental chamber (702-ASHR4, Labline Environette, Labline Instruments, Inc., Melrose Park, IL) on a slightly angled orbital shaker (Model 1000, 89032-088, VWR Scientific Products, Suwanee, GA) set speed of 2 rpm at 90% humidity and 28°C (83°F) for 24 h. The tube with the filter retained moisture at the same rate as the open tube. The tube with the loosely fitting cap retained the least amount of moisture. Therefore, the sample tubes were covered with a sterile 0.45µm filter during experimentation to allow moisture exposure but prevent bacterial aerosolization contamination during incubation of the inoculated samples.

Each inoculated and uninoculated sample was covered with a sterile 0.45µm filter affixed to the top of the open tube with a rubber band and laboratory tape. The samples were initially placed into an incubator without additional humidity for 24h. Then, samples were enumerated and subsequently placed in the environmental chamber (702-ASHR4, Labline Environette, Labline Instruments, Inc., Melrose Park, IL) set at 90% humidity at 28°C (83°F) for 48 and 72 h. The water activity of each of the samples was measured after the appropriate time interval to determine if the samples absorbed moisture or if the water activity levels stayed the same at 90% humidity. Subsamples (approximately 2-3 g) were aseptically collected in duplicate from the inoculated and uninoculated samples with sterile stainless steel spatulas (82027-532, VWR Scientific Products, Suwanee, GA) and transferred into water activity cups. The water activity cups (40107, Decagon Devices, Inc, Pullman, WA) were sealed with parafilm (52858-032, VWR Scientific Products, Suwanee, GA) and stored at room temperature in sealed, 1.5-gallon, Rubbermaid® storage container until water activity was measured and recorded. Additionally, 1 g subsamples of each uninoculated and inoculated sample was aseptically transferred with sterile stainless steel spatulas and transferred into sterile standard Standard Class O phosphate/magnesium chloride dilution buffer (BDH-0268-500g, VWR Scientific Products, Suwanee, GA; J364-100g, VWR Scientific Products, Suwanee, GA) at each time interval.

Dilutions were carried out to the 10-9 dilution and plated in duplicate onto XLD (90003-996, VWR Scientific Products, Suwanee, GA) (Wehr and Frank, 2004). Controls included media and diluent sterility controls as well as uninoculated rendering samples (50% fat). Plates were incubated overnight at 35ºC and enumerated.

Results :

The initial *Salmonella* Enteritidis culture concentration used to inoculate poultry and beef samples was $3.60*10^9$ CFU/g. After inoculation, enumeration of the poultry meal samples indicated an average *Salmonella* population of $1.11*10^7 \pm 9.89*10^6$ CFU/g. After 24 h without humidity, the inoculated poultry samples were enumerated and revealed an average Salmonella population of $3.11*10^5 \pm 2.13*10^5$ CFU/g. Samples were then placed into the humidity chamber set at 90% humidity at 28°C (83°F) for 48 and 72 h. At 48h and 72 h, samples were enumerated. At 48h, the average *Salmonella* population decreased to $4.69*10^5 \pm 4.80*10^5$ CFU/g. At 72 h, the average population of *Salmonella* in the poultry meal samples further decreased to $9.03*10^4 \pm 5.85*10^4$ CFU/g. The *Salmonella* population in the poultry samples decreased approximately 3 $\log_{10}$ cfu/g despite the exposure to increased humidity levels.

Enumeration of the inoculated beef meal samples indicated an average *Salmonella* population of $1.38*10^7 \pm 9.95*10^6$ CFU/g. After 24 h without humidity, the inoculated beef meal samples were enumerated and revealed an average *Salmonella* population of $2.66*10^5 \pm 1.48*10^5$ CFU/g. All samples were then placed into the humidity chamber set at 90% humidity at 28°C (83°F) for 48 and 72 h. At 48h and 72 h, samples were enumerated. At 48h, the average Salmonella population decreased to $1.26*10^5 \pm 1.87*10^4$ CFU/g. At 72 h, the average population of *Salmonella* in the beef samples only slightly decreased to $1.19E*10^5 \pm 4.08*10^4$ CFU/g. The *Salmonella* population in the beef meal samples decreased approximately two logarithmic ($\log_{10}$) cycles despite the exposure to increased humidity levels and rising water activity levels. Enumeration of the uninoculated poultry and beef meals samples indicated no growth of *Salmonella* on XLD agar plates. However, the water activity levels of the beef and poultry samples gradually increased throughout the 72 h at the 90% humidity level.

Salmonella growth decreased after inoculation but still detectable in both poultry and beef rendered meal samples when enumerated at 24, 48, and 72 h (Figures 7 and 9). Water activity levels of each poultry and beef meal samples increased with exposure to 90% humidity levels at 28°C (83°F) when subsamples were measured at 24, 48, and 72 h (Figures 8 and 10). The water activity levels increased to a range in which *Salmonella* species can survive and potential grow.

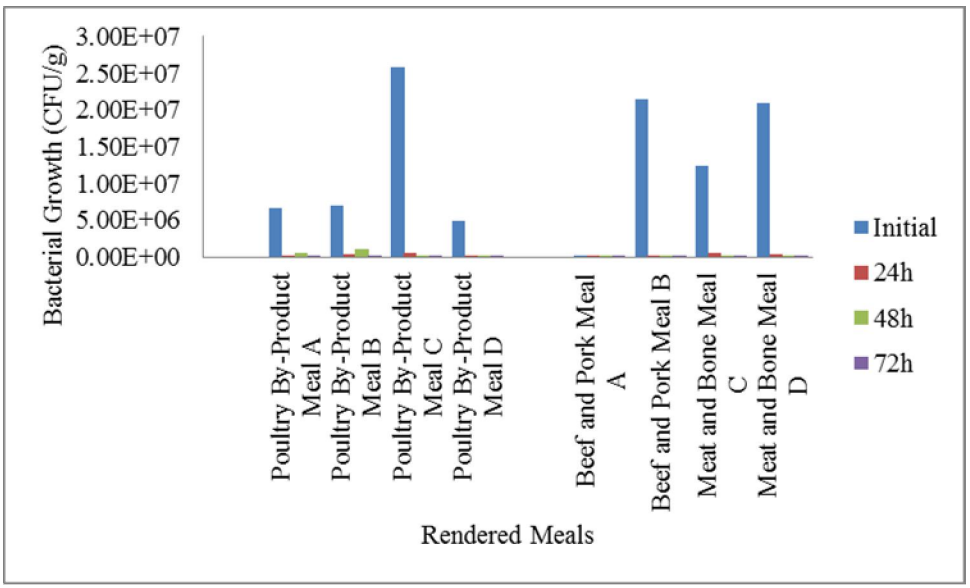

Figure 7. Growth of *Salmonella* Enteriditis (SE) in randomly selected, inoculated poultry and beef rendered meal samples upon inoculation as well as 24 (without humidity), 48, and 72 h at 90% humidity at 28°C (83°F).

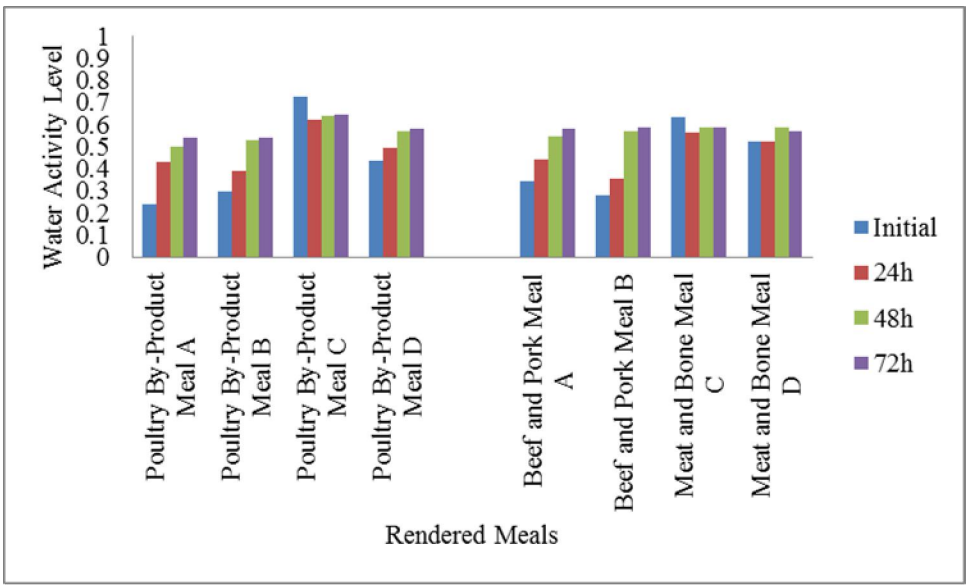

Figure 8. Water activity levels in randomly selected, inoculated poultry and beef rendered meal samples upon inoculation as well as 24 (without humidity), 48, and 72 h at 90% humidity at 28°C (83°F).

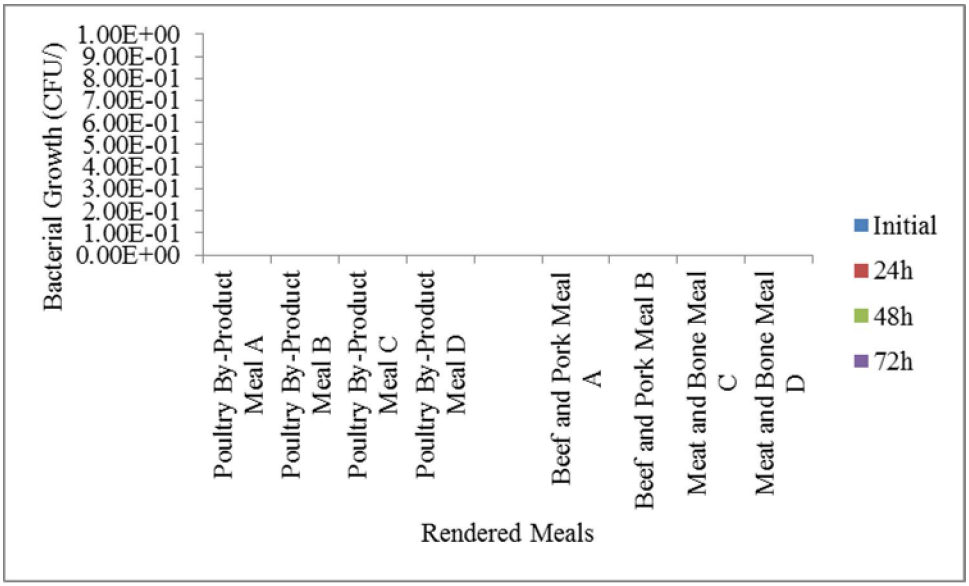

Figure 9. Growth of *Salmonella* Enteriditis (SE) in randomly selected, uninoculated poultry and beef rendered meal samples upon inoculation as well as 24 (without humidity), 48, and 72 h at 90% humidity at 28°C (83°F).

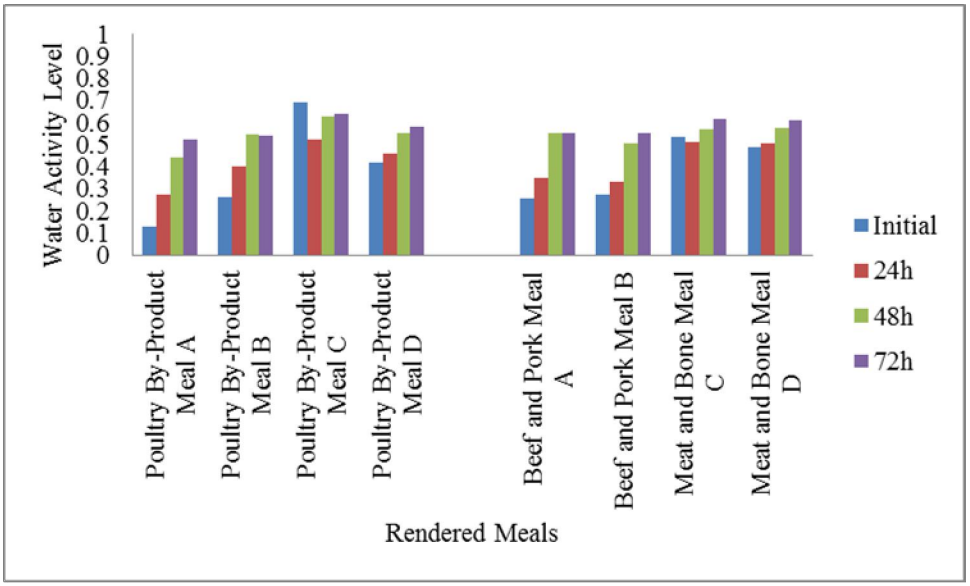

Figure 10. Water activity levels in randomly selected, uninoculated poultry and beef rendered meal samples upon inoculation as well as 24 (without humidity), 48, and 72 h at 90% humidity at 28°C (83°F).

Survival of *Salmonella* Introduced into Rendered Meals over 14 Days Storage

Introduction:

In this study, selected poultry and beef meals were analyzed for their initial water activity and then inoculated with pathogenic *Salmonella* cocktails. Survival rate was monitored on day 0, 7 and 14 at 28°C.

Materials and Methods:

Poultry and Beef Meal Sample Preparation

Twelve poultry meal samples and twelve beef meal samples were randomly chosen from samples supplied by rendering companies in the United States. Poultry samples include five poultry by-products meals, four poultry meals and three medium ash poultry meals. Beef samples include six meat and bone meals, five beef and pork meal and one blended meat and bone meal. Aqualab (Aqualab series 3TE, Decagon devices Inc, Pullman WA 99163) and sample cups (AquaLab Sample Cups 500 qty bottoms from Decagon Devices, Inc. in Pullman, WA 99163) were used to measure the water activity of these samples in duplicate. After determining the water activity and comparing to other poultry meal samples; four of these samples were in the range of lower water activity compare to other poultry meal samples, four samples were in the medium water activity range, and four samples were in the higher water activity range. Beef samples also were divided into different categories based on their initial water activity with four samples in the lower water activity range, four in the medium range and four in the higher water activity range.

Salmonella cocktail Preparation

Four serotype strains of *Salmonella* were used to make the cocktail: *Salmonella* Enteriditis, *Salmonella* Choleraesuis, *Salmonella* Newport, and *Salmonella* Dublin. Ten microliter of Each individual strain was inoculated into 1 L of the sterile Trypticase soy broth (VWR Scientific Products, Suwanee, GA) broth with 1% yeast extract (MP Biomedicals, LLC, Solon, Ohio), and incubated at 35°C overnight. After 24hr of incubation, each overnight culture was washed three times by centrifugation at 7,000 x g for 7 min (GSA rotor, DuPont RC5C Sorvall Instruments Centrifuge, DuPont Company, Newtown, CT) and resuspended in sterile physiological (0.85%) saline. The concentration of each individual strain was adjusted to 0.7 optical density (about 10^9 cfu/mL) (µQuant Universal Microplate Spectrophotometer, Bio-Tek Instruments, Winooski, VT) at 600 nm and then mixed together with the equal volume. After mixing, the *Salmonella* cocktail was placed on ice immediately. *Salmonella* cocktail for beef and poultry samples were prepared freshly on separate date.

Sample inoculation

Aluminum cans (Uline S-15743 No-handle pint cans, Pleasant Prairie, WI, 53158) and an electric paint shaker (F5 portable paint shaker, Sequoia Brands Inc, Odessa, FL 33556) were obtained. Thirty grams of each poultry and beef meal were aseptically measured into autoclave sterilized aluminum paint cans (in duplicate). All the aluminum cans were sealed aseptically, shaken on an electric paint shaker and incubated at 28°C. Samples were enumerated at 0, 7 and 14 days for *Salmonella*. A preliminary experiment on sample mixing was conducted by pipetting 0.3 mL of crystal violet (Crystal violet dye - 90008-894, VWR Scientific Products, Suwanee, GA) into the aluminum cans containing either poultry and beef meal. The cans were

sealed and shaken on a commercial paint shaker for different time intervals. Glitterbug Powder (Brevis, Salt Lake City, UT) mixed in water was also used to poultry meal to see if the inoculum could be thoroughly mixed together using the paint shaker. After observing the preliminary test results under the microscope or UV light (Brevis, Salt lake city, UT), 10 min processing of the sample cans on the paint shaker was chosen in order to achieve complete mixing.

For the inoculated samples, 0.3 mL of the *Salmonella* cocktail was aseptically inoculated into the aluminum can (Uline S-15743 no-handle pint cans, Pleasant Prairie, WI, 53158) containing pre-measured rendered meals and then immediately shaken for 10 min on the paint shaker (F5 portable paint shaker, Sequoia Brands Inc, Odessa, FL 33556). After 10 min of shaking, the aluminum can was aseptically opened and 1 gram of the inoculated poultry/beef meal was transferred into the sterile standard Class O phosphate/magnesium chloride dilution buffer. This step was repeated in duplicate.

After mixing the inoculated animal meal with the dilution buffer by shaking and vortexing (Super Mixer, 1290, Labline Instruments, Inc., Melrose Park, IL), serial dilution was made by spread plating on xylose lysine deoxycholate agar (XLD, 90003-996, VWR Scientific Products) plates. Initial *Salmonella* cocktail concentration was determined by serial dilution plating. Uninoculated samples were also plated with the same procedure. All the plates were incubated at 35°C for 24 hr. All the aluminum cans were sealed asceptically and incubated at 28°C. The same dilution plating procedure was repeated at day 7 and day 14.

Results and Discussion:

After water activity measurement, samples were divided into low, medium and high water activity category. Poultry and beef sample initial water activity are shown in Tables 2 and 3. For the poultry samples, group A, B and C had average water activity of 0.39, 0.25, and 0.23 respectively. For the beef samples, group D, E and F had average water activity of 0.54, 0.42, and 0.35 respectively. Preliminary experiments were conducted to test whether *Salmonella* cocktail inoculation would impact the water activity of both poultry and beef samples; the results indicated that inoculation of 0.3 ml of *Salmonella* cocktail into 30 gram of the rendered animal meal would not make a significant difference in water activity of the rendered animal meal.

Initial *Salmonella* cocktail population for poultry samples were 9.39±0.07 $\log_{10}$ cfu/g. At day 0, for poultry samples, group A, B, and C had average *Salmonella* concentration of 6.37, 5.56 and 6.90 $\log_{10}$ cfu/g respectively. At day 7, group A, B, and C had average *Salmonella* concentration of 2.47, 2.95 and 3.23 $\log_{10}$ cfu/g respectively. At day 14, group A, B, and C had average *Salmonella* concentration of 2.78, 3.25 and 3.21 $\log_{10}$ cfu/g respectively (Figure 10). As noted in Figure 10, the *Salmonella* population decreased an average of 3-4 $\log_{10}$ cfu/g after inoculation into rendered products despite the initial water activity level.

Initial *Salmonella* cocktail population for beef samples were 9.17±0.04 $\log_{10}$ cfu/g respectively. At day 0, for beef samples, group D, E, and F had average *Salmonella* concentrations of 6.67, 6.18 and 5.95 $\log_{10}$ cfu/g respectively. At day 7, group D, E, and F had average *Salmonella* concentrations of 3.63, 3.50 and 3.42 $\log_{10}$ cfu/g respectively. At day 14, group D, E, and F had average *Salmonella* concentrations of 2.88, 2.80 and 3.38 $\log_{10}$ cfu/g respectively (Figure 11). As noted in Figure 11, the *Salmonella* population decreased an average of 3-4 $\log_{10}$ cfu/g after inoculation into rendered products despite the initial water activity level.

From day 0 to day 7 (Figure 10 and 11), inoculated *Salmonella* populations decreased from approximately 6 $\log_{10}$ to 3 $\log_{10}$ for both poultry and beef samples. The water activity for poultry samples were ranged from 0.204 to 0.501, and the water activity for beef samples ranged

from 0.322 to 0.657. The minimum reported a_w for *Salmonella* spp is 0.94 (FDA 2013) and osmotic shock can also decrease *Salmonella* population in the dry rendered animal meals.
At day 14, *Salmonella* populations of 2.78, 3.25 and 3.21 $\log_{10}$ cfu/g remained in the three groups, respectively, in the poultry meal. In beef, *Salmonella* populations of 2.88, 2.80 and 3.38 $\log_{10}$ cfu/g in the three groups remained.

The results supported previous researchers' conclusion that artificially inoculated pathogenic *Salmonella* populations decline in low water activity environments due to many factors including osmotic shock and low available water. However, at day 14 of this study, *Salmonella* was still detected in the poultry and beef meals. Further research and information is needed for more accurate correlation between water activity and *Salmonella* survival in rendered animal matrix.

Table 2. Poultry sample initial water activity before inoculating *Salmonella* cocktail. Group A has four higher water activity poultry meals, group B has four medium water activity poultry meal, and group C has four lower water activity poultry meal.

Sample No.	Product type	Initial water activity a_w
A1	Medium ash poultry meal	0.501
A2	Poultry by-product meal	0.3725
A3	Poultry meal	0.356
A4	Poultry meal	0.3515
B1	Poultry meal	0.2545
B2	Poultry meal	0.2525
B3	Medium ash poultry meal	0.248
B4	Poultry by-product meal	0.246
C1	Poultry meal	0.239
C2	Medium ash poultry meal	0.233
C3	Poultry by-product meal	0.2295
C4	Poultry meal	0.204

Table 3. Beef sample initial water activity before inoculating *Salmonella* cocktail. Group D has four higher water activity beef meals, group E has four medium water activity beef meal, and group F has four lower water activity beef meal.

Sample No.	Product type	Initial water activity a_w
D1	Meat and bone meal	0.657
D2	Meat and bone meal	0.517
D3	Meat and bone meal	0.503
D4	Meat and bone meal	0.499
E1	Meat and bone meal	0.485
E2	Meat and bone meal	0.444
E3	Beef and pork meal	0.390
E4	Beef and pork meal	0.3725
F1	Beef and pork meal	0.364
F2	Blended MBM	0.353
F3	Beef and pork meal	0.353
F4	Beef and pork meal	0.322

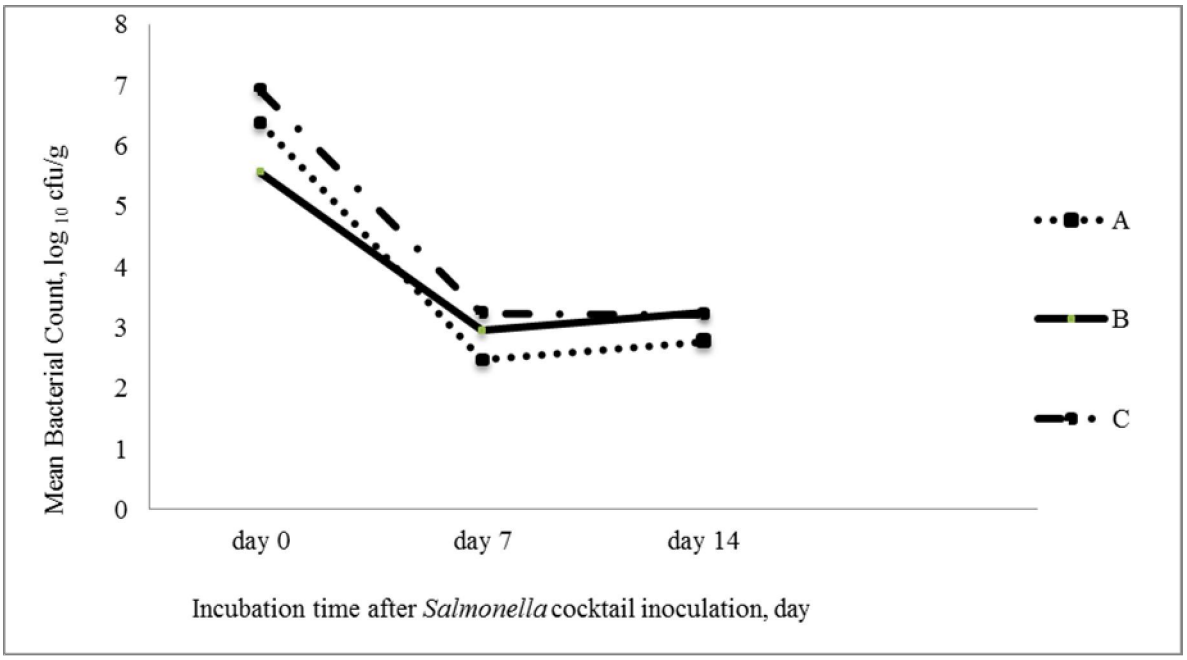

Figure 10. Poultry samples with inoculated *Salmonella* cocktail at day 0, day 7 and day 14. Initial *Salmonella* cocktail population for poultry samples were 9.39±0.07 $\log_{10}$ cfu/g. A is the group average of four poultry samples with higher initial water activity, B is the group average of four poultry samples with medium water activity, and C is the group average of four poultry samples with low water activity.

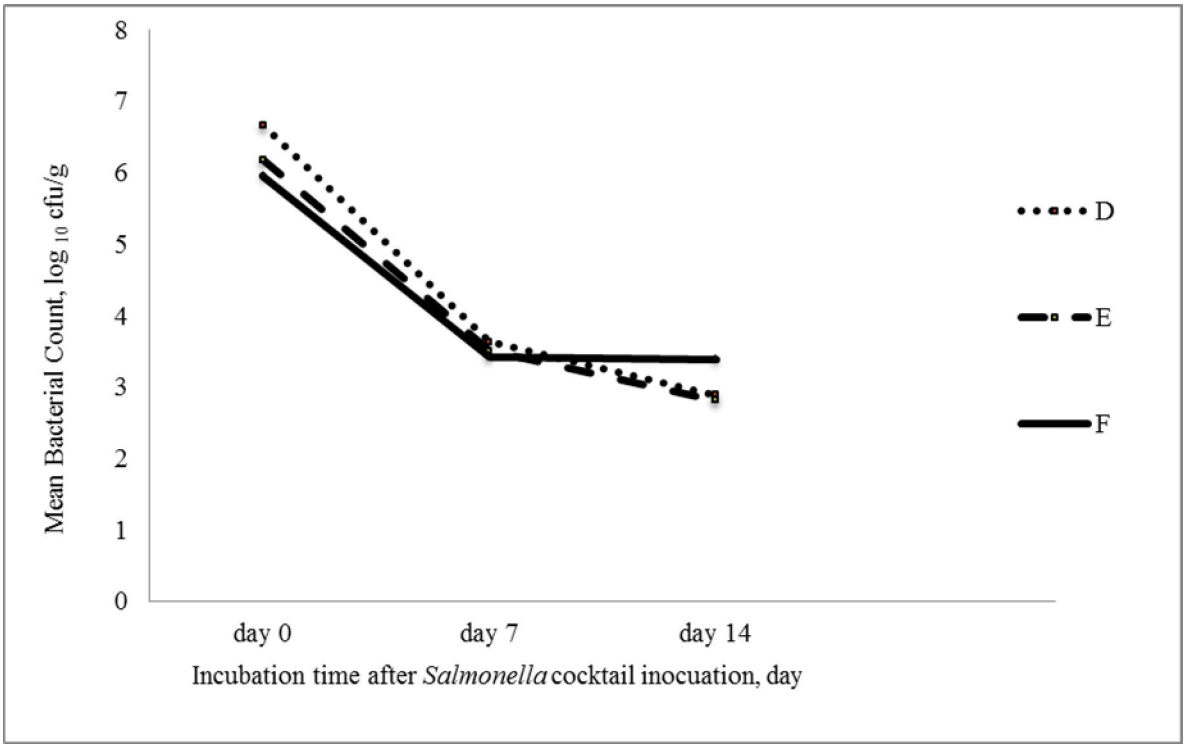

Figure 11. Beef samples with inoculated *Salmonella* cocktail at day 0, day 7 and day 14. Initial *Salmonella* cocktail population for beef samples were 9.17±0.04 $\log_{10}$ cfu/g. D is the group average of four beef samples with higher initial water activity, E is the group average of four beef samples with medium water activity, and F is the group average of four beef samples with low water activity.

Survival of *Salmonella* Cocktail in Rendered Meals with Water Activity Adjusted to 0.7, 0.8 and 0.9

Introduction:

In this experiment, several poultry and beef rendered animal meals were selected and adjusted to 0.7, 0.8 and 0.9 water activity. A four serotype *Salmonella* cocktail was inoculated into these samples and incubated at 28°C for 0 hr, 24hr, 48hr and 96hr.

Materials and Methods:

Poultry and beef meal sample preparation

Four poultry meal samples and four beef meal samples were randomly chosen from samples supplied by rendering facilities in the United States. An Aqualab water activity device (Aqualab series 3TE, Decagon devices Inc, Pullman WA 99163) and sample cups (AquaLab Sample Cups 500 qty bottoms from Decagon Devices, Inc. in Pullman, WA 99163) were used to measure the water activity of these samples in duplicate. After determining the water activity and comparing to other poultry meal samples, two higher initial water activity and two lower water activity samples were selected. Two poultry by-products meals and two medium ash poultry meals were selected. For the beef samples, two higher water activity and two lower water activity samples were selected, including two beef and pork meal and two meat and bone meals. After recording their initial water activity, each of the beef and poultry samples were adjusted to approximately a_w of 0.7, 0.8 and 0.9 by adding sterile ddH_2O. Preliminary experiments had been conducted to test the amount of water needed in order to reach a_w of 0.7, 0.8 and 0.9 with poultry and beef samples, respectively.

Salmonella cocktail preparation

Four serotype strains of *Salmonella* were used to make the cocktail: *Salmonella* Enteriditis, *Salmonella* Choleraesuis, *Salmonella* Newport, and *Salmonella* Dublin. Ten microliter of each individual strain was inoculated into 1 L of the sterile Trypticase soy broth (VWR Scientific Products, Suwanee, GA) broth with 1% yeast extract (MP Biomedicals, LLC, Solon, Ohio), and incubated at 35°C overnight. After 24hr of incubation, each overnight culture was washed three times by centrifugation at 7,000 x g for 7 min (GSA rotor, DuPont RC5C Sorvall Instruments Centrifuge, DuPont Company, Newtown, CT) and resuspended in sterile physiological (0.85%) saline. The concentration of each individual strain was adjusted to 0.7 optical density (about 10^9 cfu/mL) (μQuant Universal Microplate Spectrophotometer, Bio-Tek Instruments, Winooski, VT) at 600 nm and then mixed together with the equal volume. After mixing, the *Salmonella* cocktail was placed on ice immediately.

Sample inoculation

Preliminary experiment on sample mixing was conducted by inoculating 0.1 mL of crystal violet (Crystal violet dye - 90008-894, VWR Scientific Products, Suwanee, GA) into the 45 ml Falcon® tubes with 10 gram of poultry/beef meal and then vortexed on the fast setting for approximately 1 min for thoroughly mixing. For the inoculated samples, 0.1 mL of the *Salmonella* cocktail was aseptically inoculated into the Falcon® tube then immediately vortexed for 1 min. After vortexing, the Falcon® tube was aseptically opened and 1 gram of the

inoculated poultry/beef meal was transferred into the sterile standard Class O phosphate/magnesium chloride dilution buffer in duplicate. After mixing the inoculated animal meal with the dilution buffer by shaking and vortexing, tubes were incubated at 28°C for 0 day, 24hr, 48hr and 96hr. Serial dilutions were made by spread plating on xylose lysine deoxycholate agar (XLD, 90003-996, VWR Scientific Products) plates. Initial *Salmonella* cocktail concentration was determined by serial dilution plating. Uninoculated samples with initial water activity and adjusted a_w of 0.7, 0.8 and 0.9 were plated with the same procedure. All the XLD plates were incubated at 35°C for 24 hr.

Results and Discussion:

After initial water activity measurement, samples were divided into low and high water activity category. Poultry and beef sample initial and adjusted water activity are shown in Tables 4 and 5. Preliminary experiments had been conducted to test whether *Salmonella* cocktail inoculation will impact the water activity of both poultry and beef samples, and the data showed that inoculation of 0.1 ml of *Salmonella* cocktail into 10 gram of the rendered animal meal did not have significant different in water activity of the rendered animal meal.

In all samples, populations of *Salmonella* slowly decreased with time in the 0.7, 0.8 and 0.9 water activity samples (Figures 12-15).

Table 4. Poultry sample water activity before inoculating *Salmonella* cocktail. Group A and B had four higher water activity poultry meals; group C and D had four medium water activity poultry meal. Initial means the initial water activity. 0.7, 0.8 and 0.9 means the adjusted water activity level.

Sample No.	Product type	Actual Measured Water activity a_w
A Initial	Medium ash poultry meal	0.69
A 0.7	Medium ash poultry meal	0.708
A 0.8	Medium ash poultry meal	0.873
A 0.9	Medium ash poultry meal	0.92
B Initial	Poultry by-product meal	0.416
B 0.7	Poultry by-product meal	0.68
B 0.8	Poultry by-product meal	0.827
B 0.9	Poultry by-product meal	0.921
C Initial	Medium ash poultry meal	0.262
C 0.7	Medium ash poultry meal	0.713
C 0.8	Medium ash poultry meal	0.805
C 0.9	Medium ash poultry meal	0.914
D Initial	Poultry by-product meal	0.1285
D 0.7	Poultry by-product meal	0.683
D 0.8	Poultry by-product meal	0.815
D 0.9	Poultry by-product meal	0.92

Table 5. Beef sample water activity before inoculating *Salmonella* cocktail. Group E and F had four higher water activity beef meals, group G and H had four medium water activity beef meal. Initial means the initial water activity. 0.7, 0.8 and 0.9 means the adjusted water activity level.

Sample No.	Product type	Water activity a_w
E Initial	Meat and bone meal	0.536
E 0.7	Meat and bone meal	0.769
E 0.8	Meat and bone meal	0.838
E 0.9	Meat and bone meal	0.92
F Initial	Meat and bone meal	0.49
F 0.7	Meat and bone meal	0.78
F 0.8	Meat and bone meal	0.85
F 0.9	Meat and bone meal	0.91
G Initial	Beef and pork meal	0.27
G 0.7	Beef and pork meal	0.739
G 0.8	Beef and pork meal	0.838
G 0.9	Beef and pork meal	0.927
H Initial	Beef and pork meal	0.256
H 0.7	Beef and pork meal	0.77
H 0.8	Beef and pork meal	0.838
H 0.9	Beef and pork meal	0.92

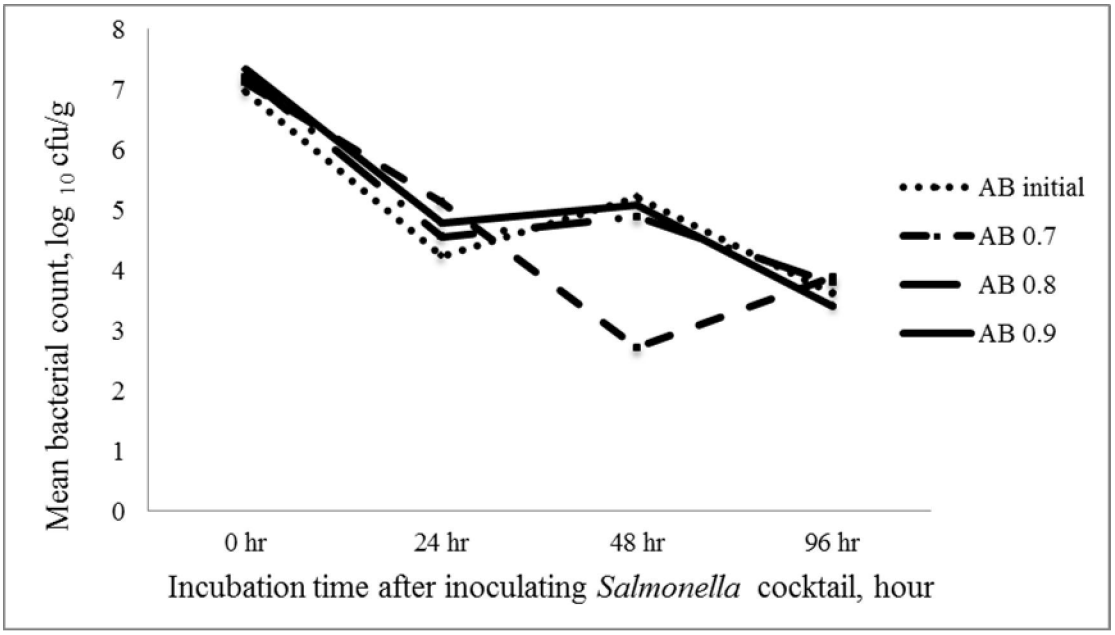

Figure 12. Poultry A and B sample average bacterial count with inoculated *Salmonella* cocktail at 0 hr, 24 hr, 48 hr and 96 hr. Initial *Salmonella* cocktail population for poultry samples were 9.55±0.06 $\log_{10}$ cfu/g. A and B poultry samples were the selected sample with higher initial water activity.

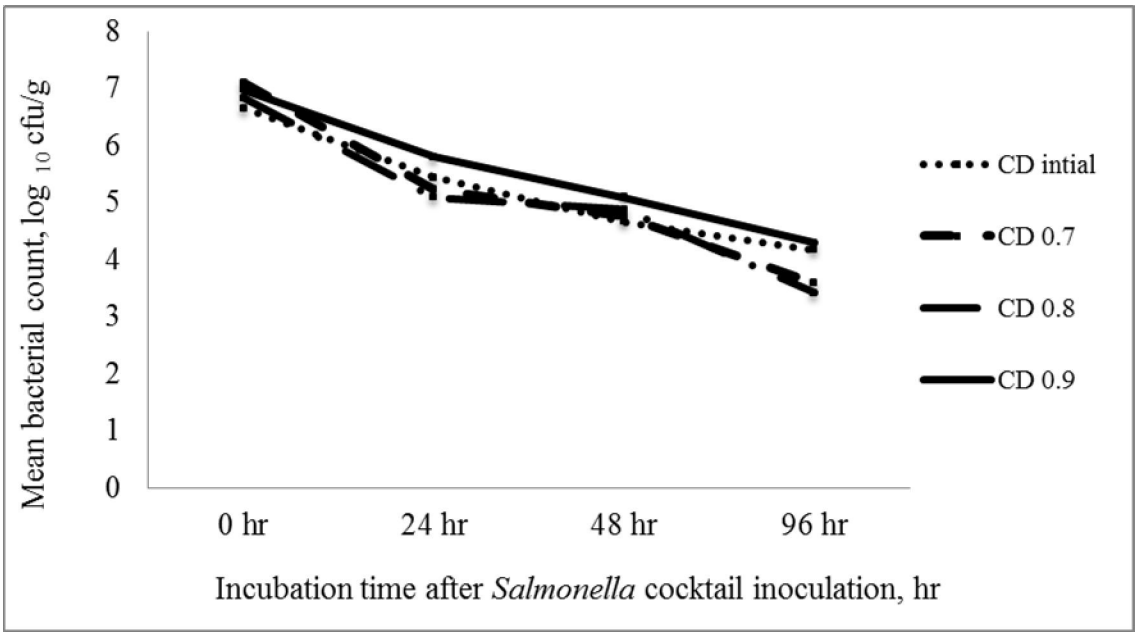

Figure 13. Poultry C and D sample average bacterial count with inoculated *Salmonella* cocktail at 0 hr, 24 hr, 48 hr and 96 hr. C and D poultry samples were the selected sample with lower initial water activity.

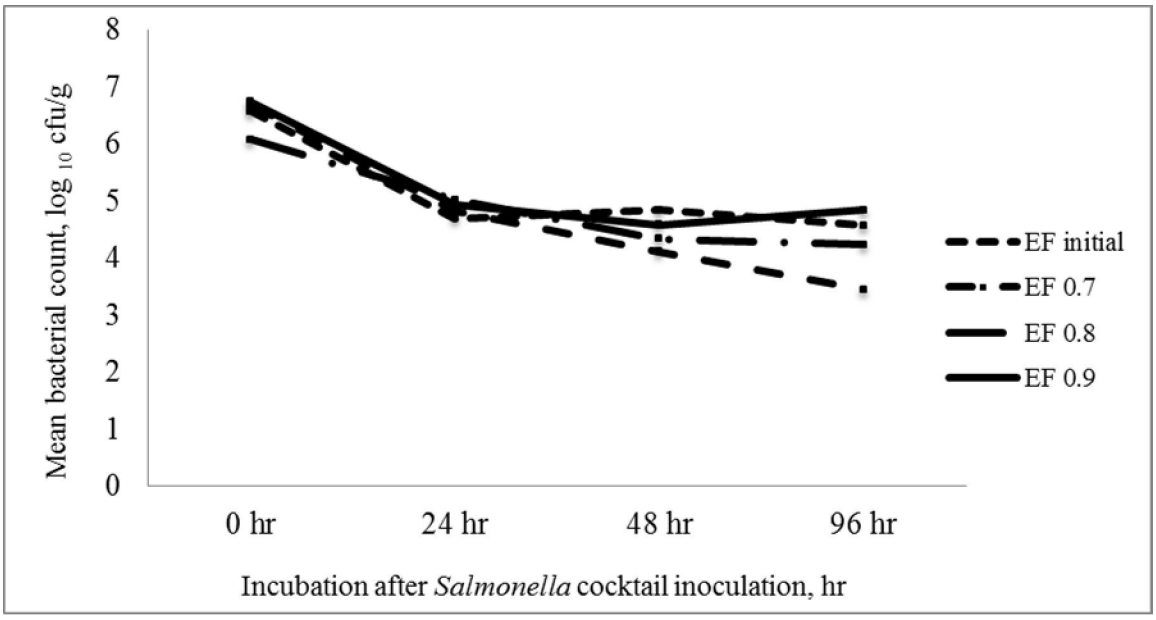

Figure 14. Beef E and F sample average bacterial count with inoculated *Salmonella* cocktail at 0 hr, 24 hr, 48 hr and 96 hr. Initial *Salmonella* cocktail population for beef samples were 9.55±0.06 $\log_{10}$ cfu/g. E and F beef samples were the selected sample with higher initial water activity.

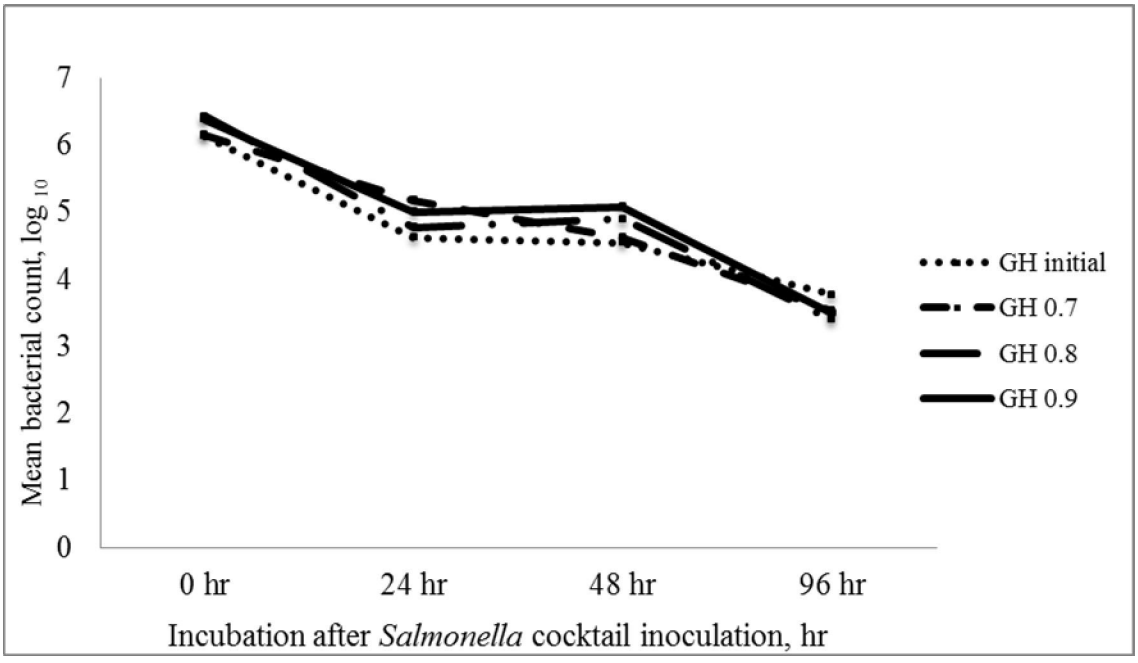

Figure 15. Beef G and H sample average bacterial count with inoculated *Salmonella* cocktail at 0 hr, 24 hr, 48 hr and 96 hr. G and H beef samples were the selected sample with lower initial water activity.

Conclusion:

Low water activity causes slow decrease in populations of *Salmonella* in rendered animal products.

References

Barbosa-Canovas, G.V., Fontana, A.J., Schmidt, S.J., and Labuza T.P. 2008. Water activity in Foods: Fundamentals and Applications. *In* IFT Press and Blackwell publishing. Imes, Iowa. USA.

Beuchat, L.R. 2009. Behavior of *Salmonella* in Foods with Low Water Activity. International Association for Food Protection Rapid Response Symposium. *Salmonella* in Peanut Butter Products. March 26, 2009. Arlington, VA. http://www.foodprotection.org/files/rr_presentations/RR_06.pdf

Burr, W.E., and Helmboldt, C.F. 1962. *Salmonella* species contaminants in three animal by-products. Avian Dis. 6: 441-443.

Carlson, T.R., Marks, B.P., Booren, A.M., Ryser, E.T., and Orta-Ramirez, A. 2005. Effect of water activity on thermal inactivation of *Salmonella* in ground turkey. J.Food Sci. 70 :7-10.

Chattopadhyay, M.K .2006 Mechanism of bacterial adaptation to low temperature; J. Biosci. 31: 157–165.

Christian, J. H. B. and Scott, W. J. 1953. Water relations of Salmonellae at 30°C. Aust. J. Biol. Sci. 6: 565-573.

Dilbaghi, N. and S. Sharma. 2007. Food and Industrial Microbiology: Food spoilage, food infections and intoxications caused by microorganisms and methods for their detection. Available from http://nsdl.niscair.res.in/jspui/bitstream/123456789/386/2/FoodSpoilage.pdf [accessed 27 August 2014].

Isa, J.M., Boycott, B.R., and Broughton, E. 1963. A survey of Salmonella contamination in animal feed and feed constituents. Can Vet Journal. 4:2.

Horton, A.J., Hak, K.M., Steffan, R.J., Foster, J.W., and Bej, A.K.2000. Adaptive response to cold temperatures and characterization of cspA in *Salmonella* typhimurium LT2. Antonie van Leeuwenhoek 77: 13–20.

Kotzekidou, P. 1998. Microbial stability and fate of *Salmonella* Enteritidis in halva, a low-moisture confection. Journal of Food Protection. 61:181-185.

Liu, T.S., Snoeyenbos, G.H., and Carlson, V.L. 1969. Thermal resistance of *Salmonella* senftenbery 775W in dry animal feeds. Avian Dis. 13: 611-631.

Maisnier-Patinm S. and Richard, J. 1996. Cell wall changes in nisin-resistant variants of *Listeria innocua* grown in the presence of high nisin concentrations. FEMS Microbiol Lett. 140 (1): 29-35.

Mossel, D.A.A., Jongerius, E., and Koopman, M.J. 1965. Sur la nécessité d'une revivification préalable pour le dénombrement des Enterobacteriaceae dans les aliments déshydratés, irradiés ou non. Ann. Inst. Pasteur Lille. 16: 119–125.

Podolak R., Enache E., Stone W., Black D. G., Elliott P. H. (2010). Sources and risk factors for contamination, survival, persistence, and heat resistance of *Salmonella* in low-moisture foods. J. Food Prot. 73: 1919–1936.

Tysset, C., Durand, C., 1973. On the survival of some gram negative, non-sporulated bacteria in commercial honey. Bull. Acad. Vet. Fr. 46: 191–196.

United States Food and Drug Administration (FDA). Bad bug book.2012. Food-borne pathogenic microorganisms and natural toxins handbook. United States Department of Human and Health Services. United States FDA. Rockville, M.D. Available from http://www.fda.gov/downloads/Food/FoodborneIllnessContaminants/UCM297627.pdf [accessed 27 August 2014].

United States Food and Drug Administration (FDA). 2013. Compliance policy guide sec. 690.800. *Salmonella* in food for animals. United States FDA. Rockville, M.D. Available from https://www.federalregister.gov/articles/2013/07/16/2013-16975/compliancepolicy-[accessed 05 August 2014].

Wehr, M. and J.F. Frank. 2004. Standard Methods for the Examination of Dairy Products. American Public Health Association, Washington, D.C.

Acknowledgments: The authors thank the rendering companies who provided the samples. Sincere appreciation is also expressed to Mr. Laine Chambers for assistance throughout the project and to Ellie Jennings and Matthew Garrett for laboratory assistance. The authors also thank Dr. Xiuping Jiang for use of her equipment as well as her insights and assistance.