The Comparative Efficacy of Hypobromous Acid from DBDMH and HBr/NaOCl against *E. coli* O157:H7 and *Salmonella typhimurium*

Background

Meat and poultry processing facilities are adopting new and improved chemical intervention steps of treating their products with FDA approved sanitizers as part of their GMP and HACCP programs. The contamination of food products by pathogenic organisms such as *Salmonella typhimurium* or *E.coli* O157 H:7 is an on-going problem that is addressed within these processing facilities using antimicrobial products. The efficacy of these Food Contact Substances (FCS) is important to assure a safe and reliable food supply. *Salmonella spp.* are the primary pathogens of interest in most chicken processing plants. As little as 100 *Salmonella* bacteria cells can cause hemorrhagic colitis, depending on the species. *Escherichia coli (E. coli)* are pathogenic bacteria that belong to the Enterobacteriaceae family. Many members of this family are a normal part of the gut flora found in the intestines of humans and other animals, but may cause serious illness when ingested. *E.coli* O157 H:7 is the most virulent strain of the *E. coli* species and causes hemorrhagic colitis at an estimated infectious dose as little as 10 – 100 bacteria cells.

DBDMH is a common antimicrobial used in meat and poultry processing facilities. It is well known that DBDMH, when dissolved in water, combines with water to form hypobromous acid, which is the sole active ingredient of DBDMH. It is a very sparingly soluble, dry compound that dissolves very slowly and is difficult to use in a production environment without use of sophisticated and complex chemical feed equipment to dissolve the material. Enviro Tech Chemical Services, Inc. recently applied fo a food-contact substance (FCN 000944) notification for an antimicrobial water treatment (processing aid), HB2, for use on meat and poultry. The FCS is hypobromous acid, which is generated on-site by mixing hydrogen bromide and a hypochlorite (such as sodium hypochlorite) to a water source. The hypobromous acid released during the use of DBDMH is **exactly** the same chemical compound as proposed in FCN #944. Not only is the DBDMH more difficult to work with, but processors can not recycle the water dosed with DBDMH in poultry chill tanks because as DBDMH decomposes it breaks down to DMH which accumulates and overstabilizes the bromine.

This report is a compilation of several experiments comparing the efficacy of hypobromous acid and hypobromous acid with excess DMH (to simulate the use of DBDMH in a recycled water environment). As stated above, the active ingredient, hypobromous acid, is the same in HB2 and DBDMH and therefore for convenience reasons, one chemical, HB2, was used for the active ingredient throughout the studies.

The experiments discussed in this report are as follows:

1. *E. coli* O157:H7 vs. HB2 and HB2 with DMH (beef study)
2. *E. coli* O157:H7 and 5% FBS (Fetal Bovine Serum) vs. HB2 and HB2 with DMH (beef study)
3. *Salmonella typhimurium* and 5% Chicken Serum vs. HB2 and HB2 with DMH
4. *Salmonella typhimurium* Inoculated Chicken Halves vs. HB2 and HB2 with DMH

Methods

Inoculum Preparation:
A wild field strain of *E. coli* O157:H7 bacteria was cultured at 35 ^{o}C in Sigma Nutrient Broth (Sigma, St. Louis, MS) for three days. Two consecutive transfers of the inoculums were made to ensure a sufficient concentration of *E. coli* O157:H7 was available for the studies. It was decided to inoculate the sample with a wild field strain of *E. coli* O157:H7 because these are considered hardier and more difficult to eradicate than those available from the laboratory institutions such as the ATCC (American Type Culture Collection). The bacteria were separated from the nutrient broth by centrifugation, and carefully resuspended in approximately 600 ml Butterfield's Buffer. The *E. coli* buffer solution was serially diluted and plated on 3M Petrifilm E.coli Plates, incubated at 35 degrees C for 48 hours where it was determined that the E. *coli* O157:H7 population was 4.27×10^8 or $\log_{10}$ 8.63 CFU/ml (Colony Forming Units per milliliter) for Experiment 1 and 1.10×10^7 or $\log_{10}$ 7.04 CFU/ml for Experiment 2

A stock solution of *Salmonella typhimurium* (ATCC 14028) was incubated at 35 degrees C for four days in Sigma Nutrient Broth for microbial culture. Three daily, consecutive transfers of the inoculums were made to ensure a sufficient concentration of *Salmonella typhimurium* was available for the study. The broth and bacteria mixture was then centrifuged leaving the *Salmonella typhimurium* to be re-suspended in approximately approximately 600 mL Butterfield's Buffer. The *Salmonella* buffer solution was serially diluted and plated on 3M Petrifilm Enterobacteriaceae Plates, incubated at 35 degrees C for 24 hours where it was determined that the *Salmonella typhimurium* population was 4.79×10^7 or $\log_{10}$ 7.68 CFU/ml for Experiment 3 and 1.45×10^6 or $\log_{10}$ 6.16 CFU/ml for Experiment 4.

A 600 ppm as Br_2 stock solution of hypobromous acid was created on-site by combining hydrogen bromide and sodium hypochlorite. This solution was split in half; one half remained unchanged and used for its nominal 600 ppm as Br_2 hypobromous acid active ingredient. DMH was added to the other half of the stock solution to yield 1440 ppm DMH and nominal 600 ppm as Br_2 hypobromous acid active ingredient. This simulates a solution that had been recycled 6 times and treated with additional DBDMH on each cycle. These solutions were used to dose the test solutions used in Experiments 1 – 3 and the two gallons used in Experiment 4.

Experiment 1: *E. coli* O157:H7 vs. HB2 and HB2 with DMH on beef

a) 100 ml *E. coli* O157:H7 stock solution to be dosed to 5 ppm Br_2 (from HB2 ONLY)
b) 100 ml *E. coli* O157:H7 stock solution to be dosed to 5 ppm Br_2 (from HB2 AND excess DMH)
c) 100 ml *E. coli* O157:H7 stock solution to be dosed to 10 ppm Br_2 (from HB2 ONLY)
d) 100 ml *E. coli* O157:H7 stock solution to be dosed to 10 ppm Br_2 (from HB2 AND excess DMH)

e) 100 ml *E. coli* O157:H7 stock solution to be dosed to 50 ppm Br_2 (from HB2 ONLY)
f) 100 ml *E. coli* O157:H7 stock solution to be dosed to 50 ppm Br_2 (from HB2 AND excess DMH)

After dosing each test solution with either the HB2 stock solution or the HB2 with extra DMH stock solution, the ppm Br_2 was immediately measured using the modified DPD colorimetric method. The solutions were plated in the same manner as above after one minute.

Experiments 2 and 3: (Experiment 2 beef; Experiment 3 chickens)

These experiments were performed the in the same manner as Experiment 1 except that higher Br_2 concentrations were used and this time it was decided that 5% serum (FBS-Fetal Bovine Serum in Experiment 2 and chicken serum in Experiment 3) be used. The efficacy of hypobromous acid, or any other antimicrobial, is compromised in the presence of organic material. Therefore the concentrations chosen in these experiments were 10 ppm, 50 ppm and 100 ppm.

a) 100 ml bacteria stock solution and 5% serum to be dosed to 10 ppm Br_2 (from HB2 ONLY)
b) 100 ml bacteria stock solution and 5% serum to be dosed to 10 ppm Br_2 (from HB2 AND extra DMH)
c) 100 ml bacteria stock solution and 5% serum to be dosed to 50 ppm Br_2 (from HB2 ONLY)
d) 100 ml bacteria stock solution and 5% serum to be dosed to 50 ppm Br_2 (from HB2 AND extra DMH)
e) 100 ml bacteria stock solution and 5% serum be dosed to 100 ppm Br_2 (from HB2 ONLY)
f) 100 ml bacteria stock solution and 5% serum to be dosed to 100 ppm Br_2 (from HB2 AND extra DMH)

After dosing each test solution with either the HB2 stock solution or the HB2 with extra DMH stock solution, the ppm Br_2 was immediately measured using the modified DPD colorimetric method. Additional measurements were taken at one and five minute time intervals. The solutions were plated in the same manner as above after one minute and five minutes.

Experiment 4:

Three whole, uncooked chickens were purchased from a local grocer. The average weight of the three chickens was 5.0 pounds. The organs were removed from each chicken and subsequently, each chicken was cut in half evenly down the back leaving six equal halves which contained a back, breast, thigh and leg. The chicken halves were then dried and marinated in the 600 ml *Salmonella* Butterfield's Buffer solution for two hours, turning occasionally. See Image 1.

Image 1

Each test solution was made with chilled water immediately prior to use. Two chicken halves were placed in each of a 12 quart-sized plastic containers and one gallon of each test solution with one disinfected ice pack was placed in the bin to accompany the chicken. The chicken halves were allowed to sit in the chilled solution for 40 minutes, turning them once and gently agitating the storage container every five minutes, see Image 2. All containers were covered using aluminum foil to prevent degradation of the active ingredients by UV light.

In summary:

a) Control: Two chicken halves- city water
b) HB2 Only: Two chicken halves- 108 ppm as Br_2
c) HB2 and DMH: Two chicken halves- 108 ppm as Br_2

Image 2

After challenge testing, the chicken half was gently shaken three times to remove excess liquid and returned to a new, sterile bag. 300g of city water was introduced to the bag and subsequently tumbled vigorously for one minute to dislodge remaining *Salmonella* bacteria. The water left at the bottom of the bag was plated using 3M Petrifilm Enterobacteriaceae Plates and incubated at 35 degrees C for 24 hours, upon which the plates were enumerated.

Results and discussion

Experiment 1: *E. coli* O157:H7 vs. HB2 and HB2 with DMH on beef

The *E. coli* O157:H7 buffer solution used in Experiment 1 contained an astonishing 4.27 x 10^8 or $\log_{10}$ 8.63 CFU/ml of *E. coli* 0157:H7 bacteria before dosing with either HB2 or the combination HB2/DMH stock solution at 5 ppm, 10ppm, and 50 ppm.. This significant amount of bacteria is believed to be responsible for the instant consumption of the active ingredient when tested immediately after dosing the solutions. The microbiological results for the control and the solutions treated with either HB2 only or the HB2/DMH solution for one minute can be seen in Table 1. When comparing the solutions dosed with either HB2 and HB2 with DMH, there were insignificant differences in terms of reduction in the number of *E. coli* O157:H7 bacteria remaining at all three concentrations tested. There was also very little difference in reduction between the 5 ppm and 10 ppm samples which were 98.42% and 98.52% for the solutions dosed with HB2 only and 97.12% and 97.66% for the solutions dosed with HB2 and DMH, respectively.

Table 1

***E. coli 0157:H7* (WITHOUT Serum) vs. HB2 ONLY**				
Description	Actual ppm Br_2 Recovered	log10 (average)	log10 reduction	% reduction
Control	N/A	8.63	N/A	N/A
5 ppm HB2 (1 min)	1.58	6.83	1.80	98.42
10 ppm HB2 (1 min)	1.80	6.80	1.83	98.52
50 ppm HB2 (1 min)	26.55	2.78	5.85	99.9998
***E. coli 0157:H7* (WITHOUT Serum) vs. HB2 & DMH**				
Description	Actual ppm Br_2 Recovered	log10 (average)	log10 reduction	% reduction
Control	N/A	8.63	N/A	N/A
5 ppm HB2 (1 min)	1.35	7.09	1.54	97.12
10 ppm HB2 (1 min)	1.76	7.00	1.63	97.66
50 ppm HB2 (1 min)	27.68	3.34	5.29	99.9994

Experiment 2: *E. coli* O157:H7 and 5% FBS (Fetal Bovine Serum) vs. HB2 and HB2 with DMH

Tables 2 and 3 and Figures 1 and 2 show the microbiological results of the control, and the samples after treatment of the HB2 solution and the HB2/DMH solution for one minute and after five minutes at nominal concentrations of 10 ppm, 50 ppm and 100 ppm as Br_2. The *E. coli* O157:H7/FBS buffer solution had a $\log_{10}$ 7.04 CFU/ml before any hypobromous acid introduction. Unlike Experiment 1, the data in Tables 2 and 3 show that there were significantly higher reductions in *E. coli* O157:H7 by the use of HB2 only compared to the samples treated with HB2 and DMH at both one minute and five minutes, indicating that the available bromine is in fact becoming overstabilized by the excess DMH in that solution and is subsequently less efficacious against the *E. coli* bacteria. At one minute there were percent reductions of 47.52 and 24.14 for the 10 ppm HB2 solution and 10 ppm HB2/DMH solutions, respectively. The same solutions resulted in a 59.26% reduction for the HB2 solution and 35.43% reduction in *E. coli* bacteria for the HB2/DMH solution after five minutes. This trend remains the same for the 50 ppm solutions. At one minute, there were percent reductions of 88.25 and 68.38 for the 50 ppm HB2 solution and 50 ppm HB2/DMH solutions, respectively. After five minutes, same solutions resulted in a 95.43% reduction for the HB2 solution and 41.12% reduction in *E. coli* bacteria for the HB2/DMH solution.

Table 2

***E. coli 0157:H7* w Fetal Bovine Serum vs. HB2 ONLY**

Description	log10 (average)	log10 reduction	% reduction
Control	7.04	N/A	N/A
10 ppm HB2 (1 min)	6.76	0.28	47.52
50 ppm HB2 (1 min)	6.11	0.93	88.25
100 ppm HB2 (1 min)	3.65	3.39	99.959

***E. coli 0157:H7 w* Fetal Bovine Serum vs. HB2 ONLY**

Description	log10 (average)	log10 reduction	% reduction
Control	7.04	N/A	N/A
10 ppm HB2 (5 min)	6.65	0.39	59.26
50 ppm HB2 (5 min)	5.70	1.34	95.43
100 ppm HB2 (5 min)	2.11	4.93	99.999

Table 3

***E. coli 0157:H7 w* Fetal Bovine Serum vs. HB2 & DMH**			
Description	log10 (average)	log10 reduction	% reduction
Control	7.04	N/A	N/A
10 ppm HB2 (1 min)	6.92	0.12	24.14
50 ppm HB2 (1 min)	6.54	0.50	68.38
100 ppm HB2 (1 min)	3.43	3.61	99.963
***E. coli 0157:H7 w* Fetal Bovine Serum vs. HB2 & DMH**			
Description	log10 (average)	log10 reduction	% reduction
Control	7.04	N/A	N/A
10 ppm HB2 (5 min)	6.85	0.19	35.43
50 ppm HB2 (5 min)	6.81	0.23	41.12
100 ppm HB2 (5 min)	2.01	5.03	99.999

Figure 1

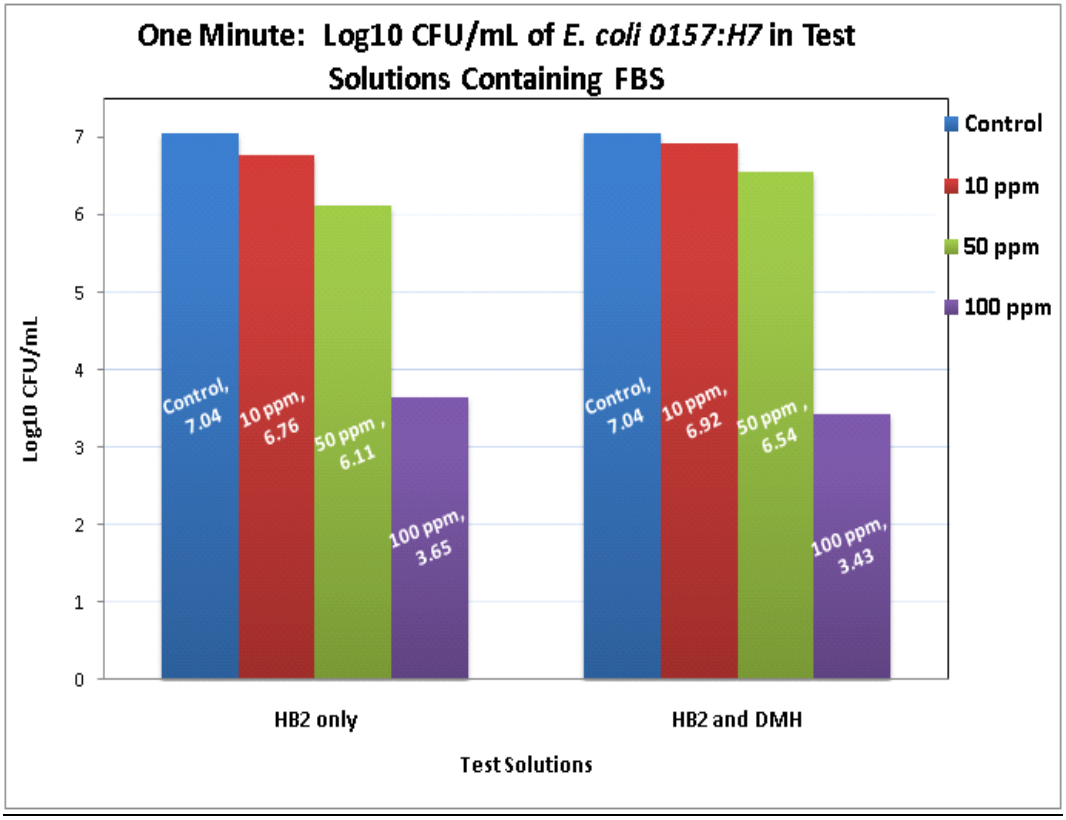
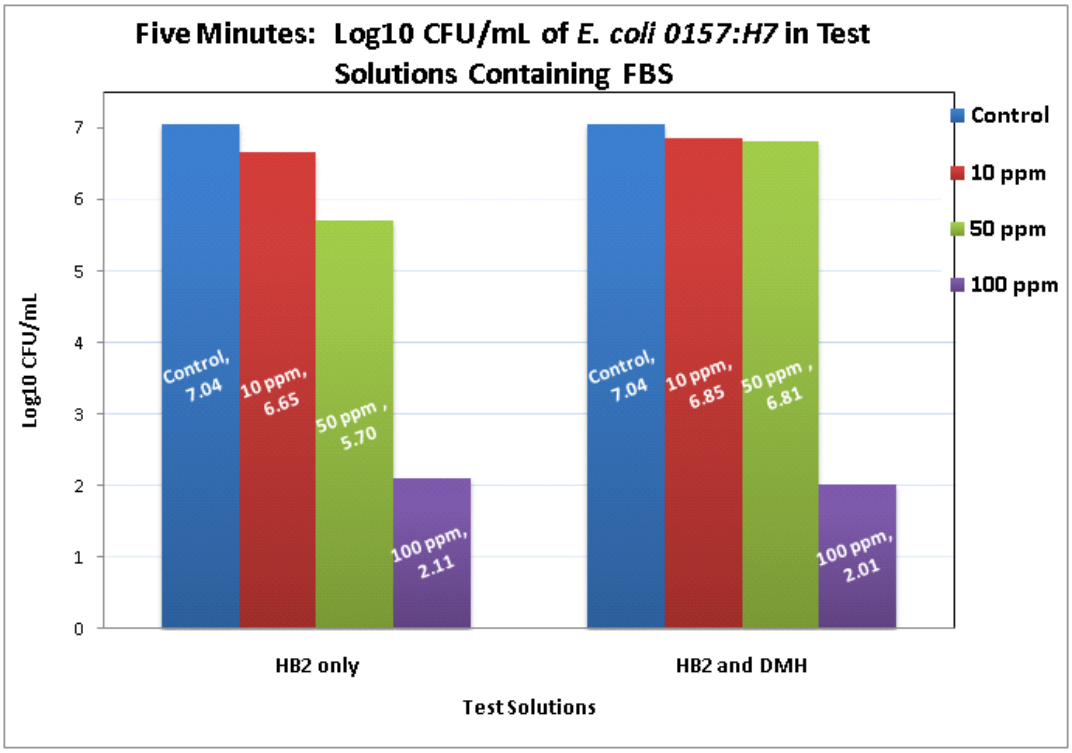
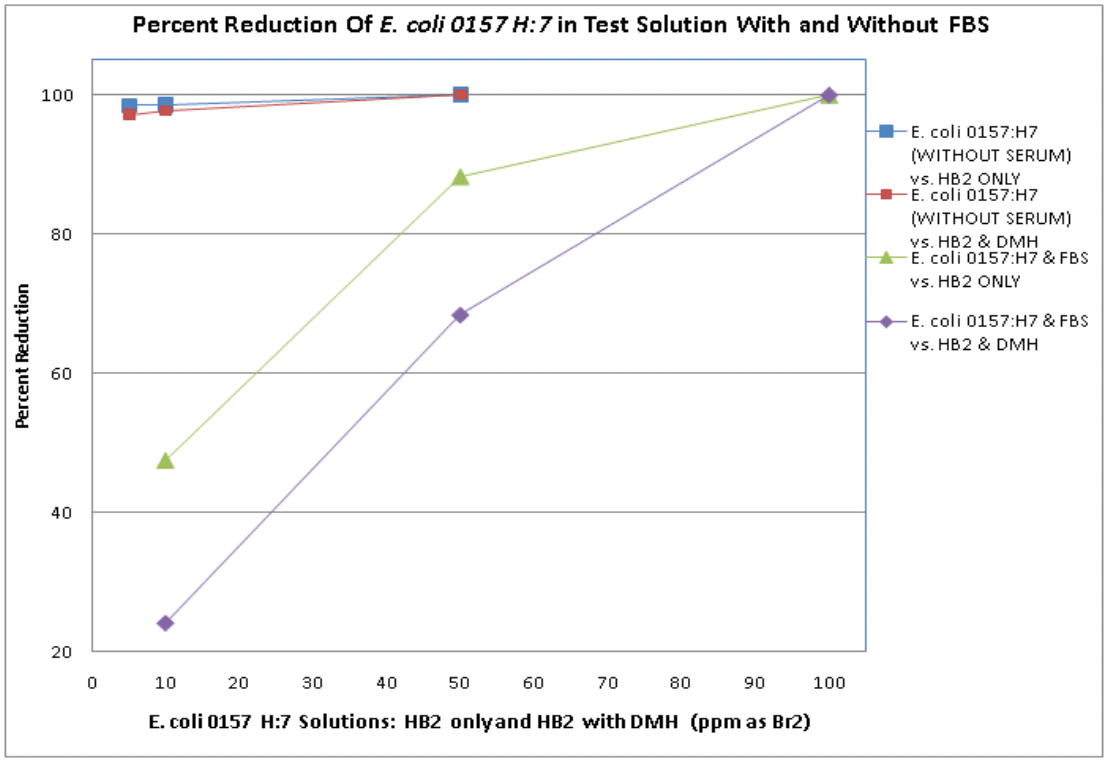


Figure 2



The bacteria buffer solutions used contained a 5 % organic load (Fetal Bovine Serum in Experiment 2 and chicken serum in Experiment 3) to represent a worst case scenario situation at a meat or poultry processing facility. It would be very rare to find 5% organic material in any meat wash water or poultry chillers. This concentration was chosen to determine the efficacy of the antimicrobial in solutions with an unusually high organic load. Figure 3 compares efficacy of both HB2 and HB2/DMH against the *E. coli* O157:H7 solutions without FBS (data from Experiment 1) and with 5% FBS (data from Experiment 2) at the three different antimicrobial concentrations. It can be seen that >99% reduction in *E.coli* occurred by the use of 50 ppm HB2 and HB2/DMH for the solutions that DID NOT contain serum. On the other hand, it took 100 ppm to eradicate >99% of the *E. coli* present in the solutions that DID contain serum. This data proves that serum present in solution has a huge demand for antimicrobials and the concentrations used should be adjusted accordingly.

Figure 3; FBS = Fetal Bovine Serum



All samples that were dosed with either HB2 only or the HB2/DMH solution were tested for residual Br_2 immediately after dosing, after one minute and after five minutes. The actual concentrations for the samples dosed to a nominal 10 ppm, 50 ppm and 100 ppm Br2 for Experiment 2 is charted, along with data from Experiment 3, in Figures 4, 5, and 6.

Figure 4

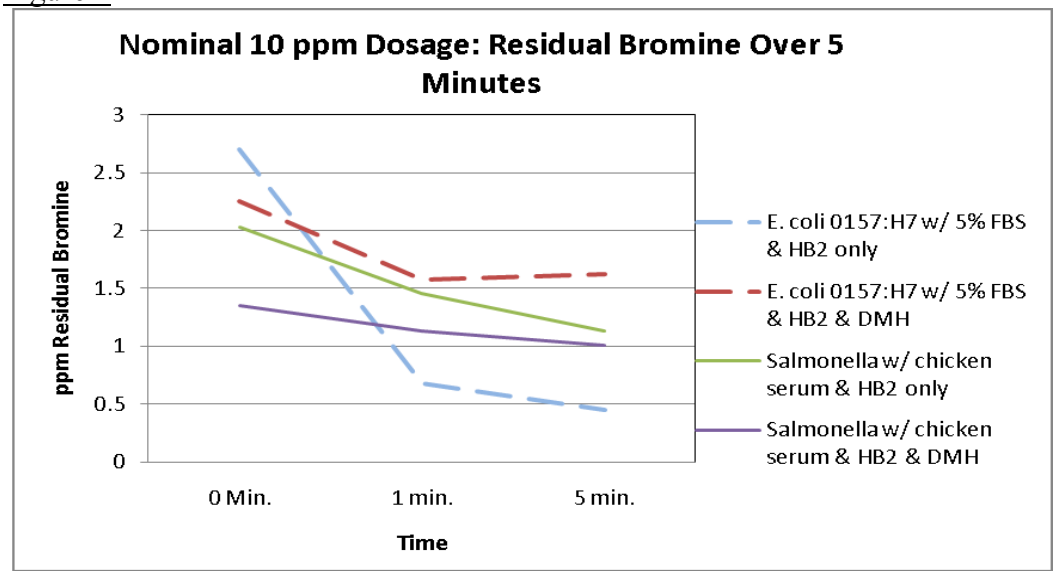


Figure 5

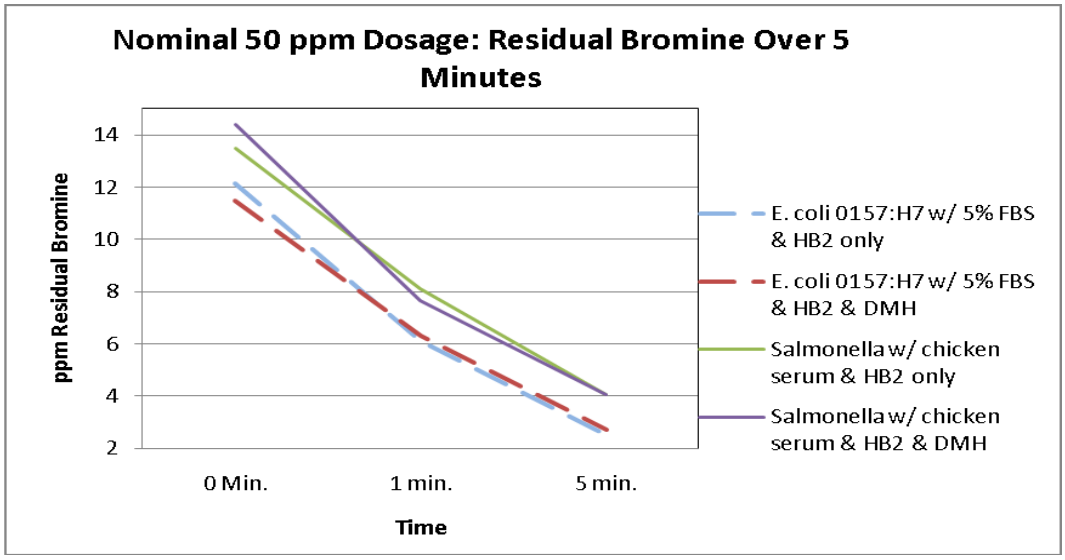
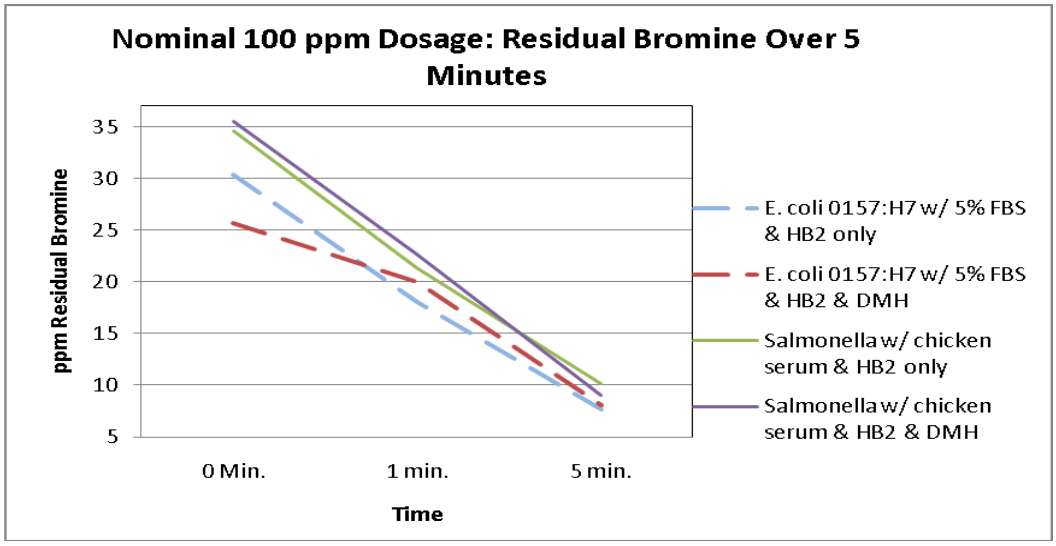


Figure 6



Experiment 3: *Salmonella typhimurium* and 5% Chicken Serum vs. HB2 and HB2 with DMH

The microbiological results of the control, and the samples after treatment of the HB2 solution and the HB2/DMH solution for one minute and after five minutes at nominal concentrations of 10 ppm, 50 ppm and 100 ppm as Br_2 can be seen in Tables 4 and 5 and Figures 7 and 8. The *Salmonella*/chicken serum buffer solution had a $\log_{10}$ 7.68 CFU/ml before any hypobromous acid was introduced. Unlike Experiment 3 which demonstrated significantly higher reductions in bacteria by the use of the HB2 solution only, the data in Tables 4 and 5 show that there was only moderately higher reductions in *Salmonella*

by the use of HB2 only compared to the samples treated with HB2 and DMH at both one minute and five minutes. At one minute there were percent reductions of 77.09 and 68.38 for the 10 ppm HB2 solution and 10 ppm HB2/DMH solutions, respectively. The same solutions resulted in an 84.86% reduction for the HB2 solution and 75.45% reduction in *Salmonella* bacteria for the HB2/DMH solution after five minutes. At one minute, there were percent reductions of 91.09 and 74.88 for the 50 ppm HB2 solution and 50 ppm HB2/DMH solutions, respectively. After five minutes, same solutions resulted in a 93.08% reduction for the HB2 solution and 95.53% reduction in *Salmonella* bacteria for the HB2/DMH solution. When using a 100 ppm solution of either HB2 or HB2 with DMH, the results are all close to a three log reduction.

Table 4

***Salmonella typhimurium* & Chicken Serum vs. HB2 ONLY**			
Description	log10 (average)	log10 reduction	% reduction
Control	7.68	N/A	N/A
10 ppm HB2 (1 min)	7.04	0.64	77.09
50 ppm HB2 (1 min)	6.63	1.05	91.09
100 ppm HB2 (1 min)	4.71	2.97	99.893
***Salmonella typhimurium* & Chicken Serum vs. HB2 ONLY**			
Description	log10 (average)	log10 reduction	% reduction
Control	7.68	N/A	N/A
10 ppm HB2 (5 min)	6.86	0.82	84.86
50 ppm HB2 (5 min)	6.52	1.16	93.08
100 ppm HB2 (5 min)	4.94	2.74	99.818

Table 5

***Salmonella typhimurium* & Chicken Serum vs. HB2 & DMH**			
Description	log10 (average)	log10 reduction	% reduction
Control	7.68	N/A	N/A
10 ppm HB2 (1 min)	7.18	0.50	68.38
50 ppm HB2 (1 min)	7.08	0.60	74.88
100 ppm HB2 (1 min)	4.71	2.97	99.893
***Salmonella typhimurium* & Chicken Serum vs. HB2 & DMH**			
Description	log10 (average)	log10 reduction	% reduction
Control	7.68	N/A	N/A
10 ppm HB2 (5 min)	7.07	0.61	75.45
50 ppm HB2 (5 min)	6.33	1.35	95.53
100 ppm HB2 (5 min)	4.36	3.32	99.952

Figure 7

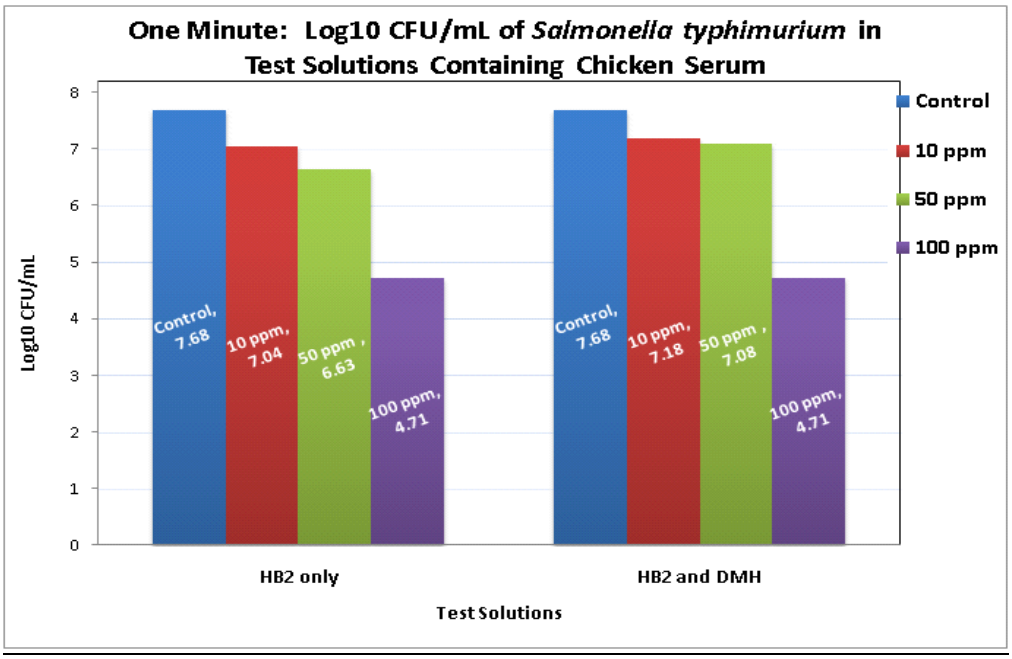
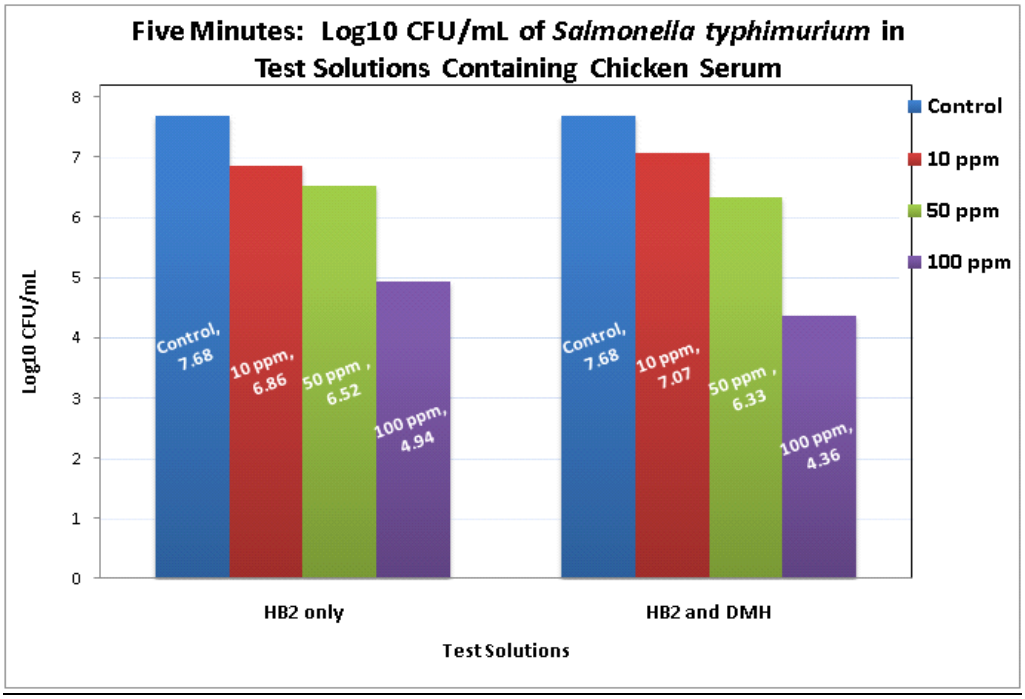


Figure 8



Experiment 4: *Salmonella typhimurium* Inoculated Chicken Halves vs. HB2 and HB2 with DMH

A higher concentration of each antimicrobial was chosen for Experiment 4, which is the submersion portion of this study, because it is required to overcome demand reactions due to the large amount of organic material present. For practical and economic reasons

the maximum concentration of 300 ppm as Br_2 from the HB2 solutions were not used, but rather a nominal 100 ppm as Br_2 was employed in this experiment because of the longer contact time and these are typical values used in poultry chillers. The actual Br_2 concentration measured by the DPD colorimetric method was determined to be 108 ppm.

Table 6 contains the average number of bacteria left on the chicken half after the 40 minute challenge test with each solution: city water (control), HB2 only, and the HB2/DMH solution. It can be seen that the control averaged a $\log_{10}$ 6.16 CFU/mL. The $\log_{10}$ reduction in *Salmonella typhimurium* bacteria, when the chicken was submerged in the HB2 solution compared to the control, was 0.23 (41.12%). The $\log_{10}$ reduction in *Salmonella typhimurium* bacteria after submerging in the HB2/DMH solution, compared to the control was 0.18 (33.93%). This data corresponds with the data from Experiments 1, 2 and 3. There was a moderately higher reduction in *Salmonella typhimurium* by the use of HB2 compared to dosing with the HB2/DMH solution. Although this data doesn't seem like the test antimicrobials are very efficacious, keep in mind that an extremely high concentration of *Salmonella typhimurium* was to be left to absorb onto the flesh of the poultry for a minimum of 2 hrs and these high levels of pathogenic bacteria are not expected to occur in actual processing facilities.

Table 6

***Salmonella typhimurium* Inoculated Chicken Halves vs. HB2 ONLY**				
Description	Actual ppm Br_2 Recovered	log10 (average)	log10 reduction	% reduction
Control	N/A	6.16	N/A	N/A
100 ppm HB2 (40 mins)	108	5.93	0.23	41.12
***Salmonella typhimurium* Inoculated Chicken Halves vs. HB2 & DMH**				
Description	Actual ppm Br_2 Recovered	log10 (average)	log10 reduction	% reduction
Control	N/A	6.16	N/A	N/A
100 ppm HB2 40 mins)	108	5.98	0.18	33.93

Conclusions

- An on going problem that meat and poultry processing facilities face is the contamination of food products by pathogenic organisms such as *Salmonella typhimurium* or *E.coli* O157:H7. It is important that these facilities use antimicrobial products that are effective in eradicating these harmful organisms to ensure a safe and reliable food supply.
- DBDMH is a common antimicrobial used in meat and poultry processing facilities. It is well known that DBDMH, when dissolved in water, combines with water to form hypobromous acid, which is the sole active ingredient of DBDMH. It is a very sparingly soluble, dry compound that dissolves very slowly and is difficult to use in a production environment. It also can not be used in recycled

water because as the DBDMH breaks down is causes an accumulation of DMH which in turn over-stabilizes the bromine.

- Enviro Tech Chemical Services, Inc. had recently applied for a FCN (#944) for HB2, which is blended on site by mixing hydrogen bromide and a hypochlorite (such as sodium hypochlorite) to a water source resulting in the active ingredient hypobromous acid. The hypobromous acid released during the use of DBDMH is **exactly** the same chemical compound as proposed in FCN Notification #944. Because the active ingredient, hypobromous acid, is the same in HB2 and DBDMH one chemical, HB2, was used for its active ingredient throughout the studies for convenience. The purpose of the excess DMH is to represent a typical scenario that might occur by the use of DBDMH after recycling. Therefore, this report should be considered a comparative confirmatory study comparing the relative efficacy of the product HB2 and DBDMH.
- Experiment 1 was the only experiment out of the four that compared the efficacy of the HB2 and HB2/DMH solutions against bacteria without the addition of organic matter. Experiments 2 and 3 involved challenging bacteria solutions that contained 5% bovine serum and Experiment 4 involved challenging *Salmonella typhimurium* inoculated chicken halves. These relatively large amounts of organic contaminants used in Experiments 2, 3 and 4 had a negative impact on the efficacy of both the HB2 and HB2/DMH. The bacteria buffer solutions used and the heavily inoculated chicken halves were used to represent a worst case scenario situation at a meat or poultry processing facility. It would be very rare to find 5% organic material in any meat wash water or poultry chillers.
- The efficacy of HB2 was greater than the HB2/DMH solution against *E.coli* 0157:H7 and *Salmonella typhimurium* in all four experiments. Although there was insignificant difference in terms of percent reduction of bacteria in Experiment 1, Experiments 2, 3 and 4 resulted in approximately 24%, 11% and 8% higher reductions by the use of HB2 only, respectively. This indicates that the available bromine is in fact becoming over stabilized by the excess DMH in the HB2/DMH solution and is subsequently less efficacious against the *E. coli* and *Salmonella* bacteria.

December 4, 2009
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