Study Title

Efficacy of High Concentrations of Hypobromous Acid Obtained from HB2 Processing Aid Sprayed on Beef Adipose Tissue Against *E. coli* O157:H7 and APC Bacteria

Data Requirements

Efficacy Data for FSIS, USDA, FDA

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Background

The contamination of meat products by pathogenic bacteria such as *E. coli* O157:H7 is of on-going concern to public health that is addressed within the processing plants by contacting the meat with antimicrobial products. The efficacy of these Food Contact Substances (FCS) is important to assure a safe and reliable food supply. Meat processing facilities are adopting new and improved chemical intervention steps for treatment of animal carcasses with FDA-approved sanitizers as part of their HACCP programs. One of these new FCS's approved for use by the FSIS (FCN 944) on February 17, 2010, is hypobromous acid (CAS No. 13517-11-8) obtained from a precursor solution of aqueous hydrogen bromide (CAS No. 10035-10-6). Under FCN 944, hypobromous acid (HOBr) is approved for use at a level not to exceed 300 ppm available bromine (133 ppm available chlorine) in water used to contact the meat products.

On the other hand, 21 CFR 173.325(c) and FCN 450 permit the use of up to 1200 ppm of sodium chlorite in solutions used to spray or dip red meat, read meat parts and organs during processing. A potable water rinse of the treated products is not required. In response to the meat processing industries' desire to use HOBr at similar elevated concentrations, Enviro Tech Chemical Services, Inc. now provides the Agency additional data to support the use of HOBr at a nominal 1000 ppm available bromine. The purpose of this study was to determine the efficacy of a 30 second spray application of HOBr (at 1000 ppm available bromine) against aerobic bacteria and *E. coli* O157:H7 which had been inoculated onto beef adipose tissue. Adipose tissue was chosen for testing since it is envisioned that HOBr solutions containing up to 1000 ppm available bromine will be applied to the external surfaces of animal carcasses which comprise mainly of adipose tissue.

Methods

Meat processing facilities commonly treat beef carcasses with antimicrobial solutions for ~30 seconds by spraying the carcasses and trim with the solution in a spray cabinet. To simulate this process, a small spray cabinet was constructed. See Image 1. A one inch air pump was used to deliver the test solution into half inch PVC tubing, which in turn allowed the test solutions to be dispensed out of six nozzles disposed four and a half inches apart in a 55 gallon drum. A regulator on the air pump was used to adjust the pressure of the spray as needed. The air pump used in this experiment can be seen in Image 2. The spray cabinet was calibrated using city water by adjusting the nozzles and pressure to ensure even distribution of the test solution. Low spray pressures were chosen so the experiment was less dependent on physical factors than on microbiological (antimicrobial) effects.

Image 1: Small Spray Cabinet

Image 2: One Inch Air Pump

As previously mentioned, beef adipose tissue was chosen as the test sample because in practice, meat processing facilities apply the antimicrobial solutions to animal carcasses which are primarily composed of adipose tissue, see Image 3. The experiment was performed by spraying ten cuts of beef adipose tissue at 25 psi for 30 seconds. The controls (potable water) and antimicrobial challenges were plated for *E. coli* O157:H7 on 3M Petrifilm E. coli Plates and aerobic bacteria on 3M APC Plates.

Image 3: Beef Adipose Tissue

The Food Contact Substance:

The Food Contact Substance that is the subject of this petition calls for the use of HOBr (at concentrations of up to1000 ppm available bromine) is the subject of this application. The HOBr was generated at the point-of-use by blending aqueous hydrogen bromide with sodium hypochlorite solution. The actual available bromine concentration measured before use in the experiment was 1047 ppm.

A stock solution of *E. coli* O157:H7 (ATCC 35150) was incubated at 35 degrees C for three days in Sigma Nutrient Broth for microbial culture. Two daily, consecutive transfers of the inoculums were made to ensure a sufficient concentration of *E. coli* O157:H7 was available for the study. The broth and bacteria mixture was then centrifuged leaving the *E. coli* O157:H7 to be re-suspended in approximately 500 mL Butterfield's Buffer. The *E. coli* inoculum 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 6.5×10^7 or $\log_{10}$ 7.81.

The study was designed for 10 replicate treatments of two pieces of adipose tissue disposed into the spray zone at the same time. Room temperature water (68F) was used throughout this experiment. One treatment was the HOBr solution and the other treatment was untreated potable water as the control. The HOBr treatment was compared to the potable water control by enumerating the amount of viable bacteria remaining on the tissue immediately (Day 0) for one piece of adipose tissue, and the amount remaining after 24 hours (Day 1) for the second piece of adipose tissue. The steps taken to accomplish this comprised the following:

1. Forty uncooked, boneless, adipose tissue pieces, of equal size were weighed. The average weight being 180 g.

   The adipose tissue pieces were placed in sterile poultry rinse bags and taken to the testing area where each adipose tissue piece was evenly sprayed with the *E. coli* inoculum. Immediately after, the spraying commenced. Ten replicate spraying of 2 pieces of adipose tissue were performed the solution of HOBr and another 10 x 2 pieces of adipose tissue was subject to an identical a potable water spray to serve as the control. Thus, 40 pieces of adipose tissue were used in the experiment.

   In summary:
   Beef Adipose Tissue:-

   i) Control: 10 x 2 pieces- 25 psi potable water- Day 0, Day 1
   ii) HOBr: 10 x 2 pieces- 25 psi 1147 ppm total bromine- Day 0, Day 1

   During each 30 second spray, the two pieces of adipose tissue were held by a hook and separated by a one inch PVC pipe and manually moved up and down while rotating to ensure even distribution of the test spray at 25 psi, see Image 4. The HOBr concentration was measured prior to spraying the pieces by using a HACH DR/700 Colorimeter and HACH 10 ml Total Chlorine pillow packets. See Addendum 1.

   Image 4: Beef Adipose Tissue Being Sprayed

2. After challenge testing, the adipose tissue pieces were gently shaken three times to remove excess liquid and returned to new, sterile bags. For the Day 0 tissue

pieces, 100 g of city water was introduced to the remaining bags and subsequently tumbled for one minute to dislodge remaining *E. coli* bacteria or aerobic bacteria. The water left at the bottom of the bag was plated using 3M Petrifilm E. coli Plates and 3M APC Plates. They were then incubated at 35 degrees C for 48 hours, upon which the plates were enumerated. All plating on Day 0 was performed within 30 minutes after the challenge testing. The Day 1 tissue pieces were placed in a refrigerator to arrest microbiological growth and then subject to the same techniques 24 hours later.

Results and Discussion

Table 1 contains the average of 10 numbers of *E. coli* O157:H7 bacteria left on the adipose tissue after being sprayed for 30 seconds with potable water (control), or HOBr solution immediately after (Day 0) and 24 hours later Day 1. It can be seen that the control averaged a $\log_{10}$ of 5.85 on Day 0 and a $\log_{10}$ 5.49 on Day 1. The $\log_{10}$ reduction in *E. coli* O157:H7 bacteria when HOBr was sprayed onto the adipose tissue, compared to the control, was 1.51 CFU/mL (96.91%) on Day 0 and 1.33 CFU/ml (95.32%) on Day 1.

Table 1: Enumeration Results of Microbiological Analysis (*E. coli O157:H7*)

Log10 CfU/ml *E.coli* O157:H7 from Adipose Tissue and Reductions

Description	Log10 (CFU/ml) Day 0	Log10 Reduction Day 0	Log10 (CFU/m1) Day 1	Log10 Reduction Day 1
Control	5.85	N/A	5.49	N/A
HOBr (1047 ppm as Br_2)	4.34	1.51 (96.91%)	4.16	1.33 (95.32%)

The average of 10 numbers of *E. coli* O157:H7 bacteria present on beef adipose tissue on Day 0 and Day 1 is charted in Figure 1.

Figure 1: Control vs. HOBr treatment. Log_{10} remaining (*E. coli* O157:H7)

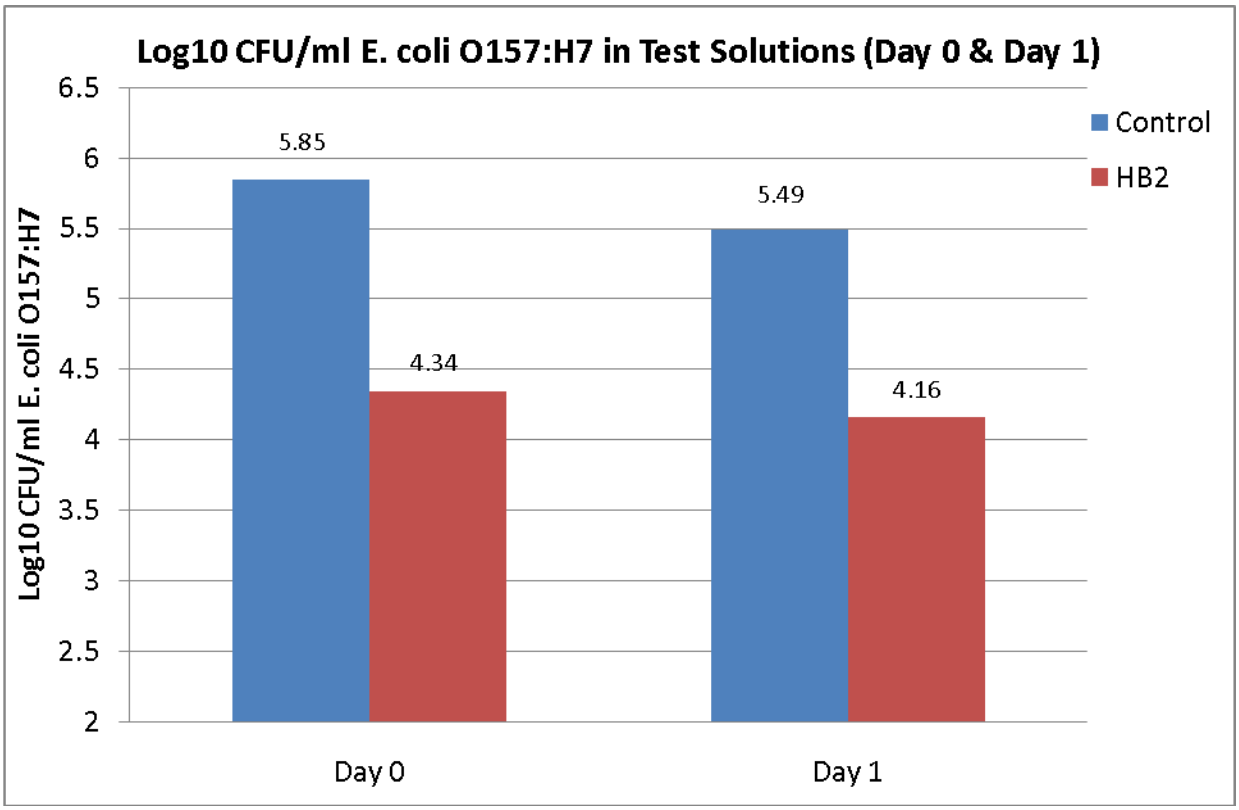


Table 2 contains the average of 10 numbers of aerobic bacteria left on the adipose tissue after being sprayed for 30 seconds with potable water (control), or HOBr solution immediately after (Day 0) and 24 hours later Day 1.The control averaged a log_{10} of 6.07 on Day 0 and a log_{10} 6.45 on Day 1. The log_{10} reduction in APC when HOBr was sprayed onto the adipose tissue, compared to the control, was 1.39 CFU/mL (95.93%) on Day 0 and 1.85 CFU/ml (98.59%) on Day 1.

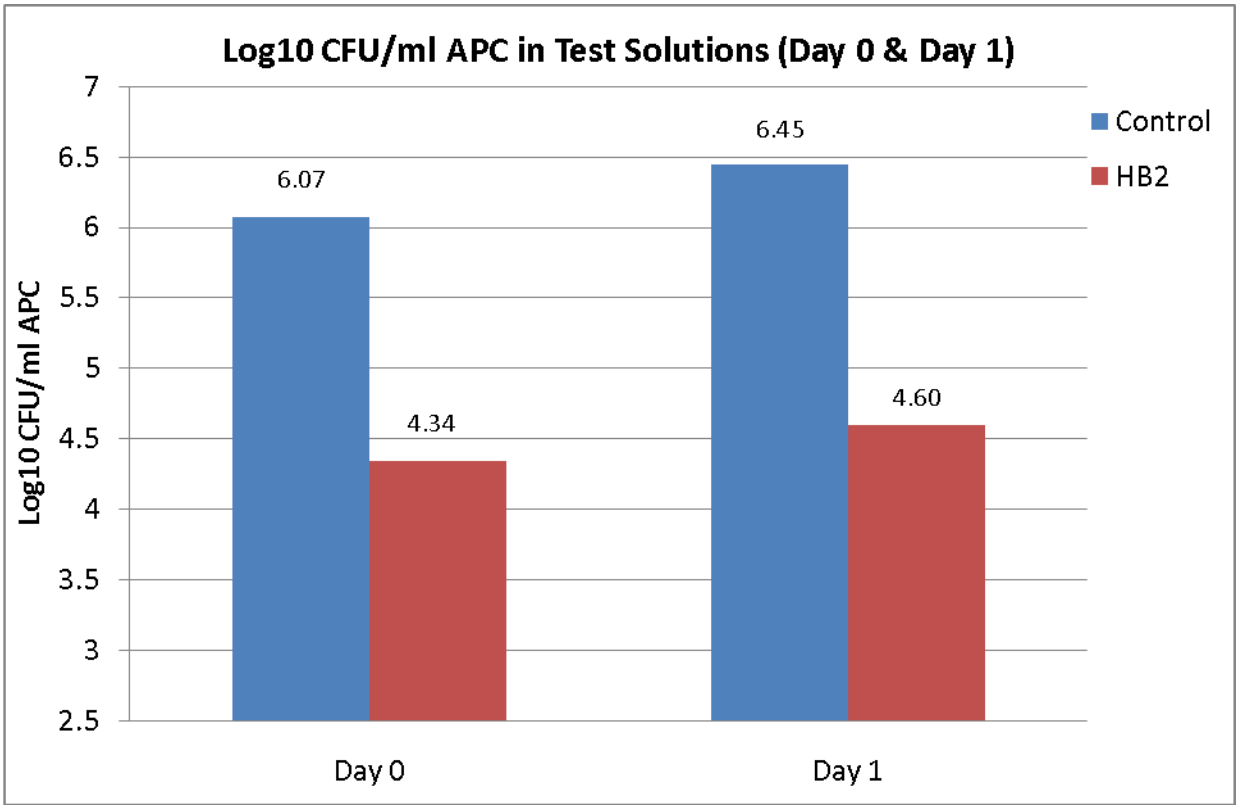
Table 2: Enumeration Results of Microbiological Analysis (APC)

Log10 CfU/ml *APC* from Adipose Tissue and Reductions

Description	Log_{10} (CFU/mL) Day 0	Log_{10} Reduction Day 0	Log_{10} (CFU/mL) Day 1	Log_{10} Reduction Day 1
Control	6.07	N/A	6.45	N/A
HOBr (1047 ppm as Br_2)	4.68	1.39 (95.93%)	4.60	1.85 (98.59%)

The average concentrations of APC present on beef adipose tissue on Day 0 and Day 1 is charted in Figure 2.

Figure 2: Control vs.HOBr treatment. Log_{10} remaining (APC)



The Student's t-test was used to assess whether the means of the microbiological results obtained in this study were significantly different from each other when comparing the control to the HOBr treated adipose tissue pieces. In doing so, the "t-test: Two Sample Assuming Equal Variances" (which is part of the Analysis ToolPak of Excel 2007) was used to perform the statistical analysis. By comparing the means of the control and the means of the HOBr treated adipose tissue pieces, the number of degrees of freedom is 18 because $(n_1 + n_2)-2$, where n_1 is 10 (the number of replicates of treatment 1), and n_2 is 10 (the number of replicates of treatment 2). Using the t-table, where the probability equals 0.05 and the degrees of freedom equal 18, the t value corresponds to 2.10. Therefore, if the t stat > t value, the means of differences are significantly different. A probability of p = 0.05 means that the level of confidence that the data are correct is equal to 95%. The data obtained in this study and seen in Addendum 2-3 show statistically significant ($p<0.05$) differences in the microbiological results obtained from the adipose tissue treated with the potable water control compared to the adipose tissue treated with HOBr for both Day 0 and Day 1.

Conclusions:

- The microbiological profile showed the potable water controls for Day 0 and Day 1 averaged a $\log_{10}$ of 5.85 and a $\log_{10}$ 5.49 of *E. coli* O157:H7, respectively. The $\log_{10}$ reduction in *E. coli* O157:H7 bacteria when HOBr (1047 ppm as Br_2) was sprayed onto the adipose tissue, compared to the control, was 1.51 CFU/ml on Day 0 (96.91% reduction) and 1.33 CFU/ml (95.32% reduction) on Day 1.
- The microbiological profile showed the potable water controls for Day 0 and Day 1 averaged a $\log_{10}$ of 6.07 and a $\log_{10}$ 6.45 aerobic plate count bacteria, respectively The $\log_{10}$ reduction in aerobic bacteria when HOBr (1047 ppm as Br_2) was sprayed onto the adipose tissue, compared to the control, was 1.39 CFU/ml (95.93% reduction) CFU/ml on Day 0 and 1.85 CFU/ml (98.59% reduction) on Day 1.

- For both sets of bacteria, there was no significant difference between the Day 0 and Day 1 results. There was no rebound in the population of either of the bacteria colonies. Nor was there any extended technical effect in terms of significantly reduced bacteria populations one day after the HOBr challenge.

- Compared to the potable water control, the HOBr solution at 1047 ppm as Br_2 displays meaningful efficacy against both *E. coli 0157:H7* and aerobic bacteria on beef adipose tissue. The short contact time scenarios used in this study are the same ones typically encountered when meat carcasses are sprayed at commercial processing facilities.

References:

Bell, Kristen Y, et al*; *"Reductions of Foodborne Micro-Organisms on Beef Carcass Tissue Using Acetic Acid, Sodium Bicarbonate, and Hydrogen Peroxide Spray Washes";* Journal of Food Microbiology, 1997, 14, 439-448.

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ADDENDUM 1

ENVIRO TECH CHEMICAL SERVICES STANDARD OPERATING PROCEDURE

Original SOP Effective Date: No Date	Superseded SOP Dated: N/A	Effective Date: 7/08/09	Procedure No.: ETQC28
Facility: Modesto	Approval Name & Signature: Tina Rodrigues		Revision No.: 1
Review Frequency: 2 years	Approval Title: Lab Manager		Page 1 of 3
Without a yellow control stamp to the right of this statement, this procedure is a draft. A draft or an uncontrolled copy cannot be used to manage a process or task.			JMM-7/9/2009
Revised Section(s): Transferred SOP to the new SOP format.			

I. TITLE: DPD ANALYSIS OF PRODUCTS USING HACH DR/700 COLORIMETER

II. PURPOSE: This document is to be used by any lab personnel involved in the analysis of products using the HACH DR/700 Colorimeter.

III. EQUIPMENT / REAGENTS:

-HACH DR/700 Colorimeter- Model number 46700-00
-DPD ***TOTAL*** Chlorine Reagent Pillow Packets (for 10mL)- number 21056-69
-Hydrogen peroxide Activator 1 (15% KI solution)
-Hydrogen Peroxide Activator 2 (5% ammonium molybdate solution)
-De-ionized or reversed osmosis water

IV. PROCEDURE:

Before testing make sure the instrument is in the low (LO) range mode by checking that the display reads to the hundredths (0.00).

1. Make an appropriate dilution if needed.

2. Fill both 10mL sample cells with 10 mLs of the water sample. Designate one of these to be the blank and the other to be the prepared sample.

Make sure the cells are not wet and they are free of fingerprints or smudges.

3. Cap the blank cell and place it in the cell holder with the diamond mark facing you. Cover the cell compartment and press ZERO. The instrument will display 0.00. Remove the blank.

4. Add the contents of one DPD Total Chlorine pillow packet to the prepared sample. Cap and shake vigorously. A pink color will develop.

5. Quickly place the sample cell in the compartment with the diamond mark facing you, close the cover and press READ.

6. The instrument display will show-- followed by the results in ppm total chlorine.

7. Note: if the instrument reads a blinking 3.67, the sample concentration is too high and needs to be diluted.

Calculations:

Total Chlorine: no calculation needed, the instrument reading is the ppm total Cl_2.

Bromine: ppm Br_2= 2.25 X total Cl_2

PAA: ppm PAA= 1.07 X total Cl_2

DBNPA: ppm DBNPA = X total Cl_2

(For DBNPA- follow steps 1 – 7 but let react 3 minutes before taking a reading)

V. PROCEDURE FOR HYDROGEN PEROXIDE ONLY

1. Make an appropriate dilution if needed.

2. Fill both 10mL sample cells with 10 mLs of the water sample. Designate one of these to be the blank and the other to be the prepared sample. Make sure the cells are not wet and they are free of fingerprints or smudges.

3. Cap the blank cell and place it in the cell holder with the diamond mark facing you. Cover the cell compartment and press ZERO. The instrument will display 0.00. Remove the blank.

4. Add 3 drops of Hydrogen Peroxide Activator 1 and 3 drops of Hydrogen Peroxide Activator 2 to the prepared sample cell.

5. Swirl the prepared sample cell and let react for 6 minutes.

6. Add the contents of one DPD Total Chlorine pillow packet to the prepared sample. Cap and shake vigorously. A pink color will develop.

7. Quickly place the sample cell in the compartment with the diamond mark facing you, close the cover and press READ.

8. The instrument display will show -- followed by the results in ppm total chlorine. This is the total Cl_2 peroxygen value.

9. Note: if the instrument reads a blinking 3.67, the sample concentration is too high and needs to be diluted.

Calculation:

ppm H_2O_2- 0.478 X (total Cl_2 peroxygen – total Cl_2 as PAA)

ADDENDUM 2: *E. coli* O157:H7 results (Day 0 & Day 1)

Day 0: Mean *E. coli* counts

	Day 0: Mean *E. coli* O157:H7 Count (Log_{10} CFU/ml)[2]		
	Test Solutions		**Log_{10} Reduction**
Adipose Sample Number	Control (Water Wash)	HOBr (1147 ppm as Br_2)	"Control vs. HOBr"[1]
1	5.84	4.37	1.47
2	5.48	4.18	1.30
3	6.08	4.45	1.63
4	6.17	4.40	1.77
5	5.70	4.19	1.51
6	6.00	3.98	2.02
7	5.51	4.47	1.04
8	6.16	4.41	1.75
9	5.71	4.43	1.28
10	5.84	4.51	1.33
Mean	**5.85**	**4.34**	**1.51**
S.D.[3]	**0.25**	**0.17**	**0.29**
C.V.[3]	**0.04**	**0.04**	**0.19**

t-Test: Two-Sample Assuming Equal Variances		
	"Control vs. HOBr" Samples	
	Variable 1	*Variable 2*
Mean	5.849	4.339
Variance	0.063187778	0.028121111
Observations	10	10
Pooled Variance	0.045654444	
Hypothesized Mean Difference	0	
df	18	
t Stat[2]	15.80230422	
P(T<=t) one-tail	2.68681E-12	
t Critical one-tail	1.734063592	
P(T<=t) two-tail	5.37361E-12	
t value	2.100922037	

[1] "Control vs. HOBr" refers to log10 difference in *E. coli* among the adipose pieces treated with Modesto city water compared to the HOBr solution at 1147 ppm as Br_2

[2] Means within a grouping with an asterisk are significantly different (P<0.05) as determined by the t-test which assesses whether the means of two groups are statistically different from each other. Therefore if t Stat > t value the means of differences are significantly different. A probability of p = 0.05 (95% probability of making a correct statement).

[3] S.D. = Standard Deviation, C.V. = Coefficient of Variation

Day 1: Mean *E. coli* counts

	Day 1: Mean *E. coli* O157:H7 Count (Log_{10} CFU/ml)[2]		
	Test Solutions		Log_{10} Reduction
Adipose Sample Number	Control (Water Wash)	HOBr (1147ppm as Br_2)	"Control vs. HOBr"[1]
1	5.05	3.83	1.22
2	4.84	5.07	-0.23
3	5.11	3.86	1.25
4	5.15	4.05	1.10
5	5.83	4.22	1.61
6	6.12	4.13	1.99
7	5.40	4.19	1.21
8	5.93	3.74	2.19
9	5.32	4.00	1.32
10	6.18	4.52	1.66
Mean	**5.49**	**4.16**	**1.33**
S.D.[3]	**0.48**	**0.39**	**0.66**
C.V.[3]	**0.09**	**0.09**	**0.49**

t-Test: Two-Sample Assuming Equal Variances

	"Control vs. HOBr" Samples	
	Variable 1	*Variable 2*
Mean	5.493	4.161
Variance	0.232801111	0.152898889
Observations	10	10
Pooled Variance	0.19285	
Hypothesized Mean Difference	0	
df	18	
t Stat[2]	6.782337629	
P(T<=t) one-tail	1.18203E-06	
t Critical one-tail	1.734063592	
P(T<=t) two-tail	2.36406E-06	
t value	2.100922037	

[1] "Control vs. HOBR" refers to log10 difference in *E. coli* among the adipose pieces treated with Modesto city water compared to the HOBr solution at 1147 ppm as Br_2

[2] Means within a grouping with an asterisk are significantly different (P<0.05) as determined by the t-test which assesses whether the means of two groups are statistically different from each other. Therefore if t Stat > t value the means of differences are significantly different. A probability of p = 0.05 (95% probability of making a correct statement).

[3] S.D. = Standard Deviation, C.V. = Coefficient of Variation

ADDENDUM 3: APC results (Day 0 & Day 1)

	Day 0: Mean APC (Log_{10} CFU/ml)[2]		
	Test Solutions		**Log_{10} Reduction**
Adipose Sample Number	Control (Water Wash)	HOBr (1147 ppm as Br_2)	"Control vs. HOBr"[1]
1	5.94	4.38	1.56
2	6.03	4.63	1.40
3	6.14	4.38	1.76
4	6.13	5.13	1.00
5	6.49	4.32	2.17
6	6.11	4.24	1.87
7	5.85	5.05	0.80
8	6.00	4.84	1.16
9	5.72	4.69	1.03
10	6.34	5.17	1.17
Mean	**6.08**	**4.68**	**1.39**
S.D.[3]	**0.22**	**0.35**	**0.44**
C.V.[3]	**0.04**	**0.08**	**0.32**

t-Test: Two-Sample Assuming Equal Variances		
	"Control vs. HOBr" Samples	
	Variable 1	*Variable 2*
Mean	6.075	4.683
Variance	0.050383333	0.123423333
Observations	10	10
Pooled Variance	0.086903333	
Hypothesized Mean Difference	0	
df	18	
t Stat[2]	10.55859228	
P(T<=t) one-tail	1.92227E-09	
t Critical one-tail	1.734063592	
P(T<=t) two-tail	3.84453E-09	
t value	2.100922037	

[1] "Control vs. HOBr" refers to log_{10} difference in APC (aerobic plate count) among the adipose pieces treated with Modesto city water compared to the HOBr solution 1147 ppm as Br_2

[2] Means within a grouping with an asterisk are significantly different (P<0.05) as determined by the t-test which assesses whether the means of two groups are statistically different from each other. Therefore if t Stat > t value the means of differences are significantly different. A probability of p = 0.05 (95% probability of making a correct statement).

[3] S.D. = Standard Deviation, C.V. = Coefficient of Variation

Day 1: Mean APC counts

	Day 1: Mean APC Count (Log_{10} CFU/ml)[2]		
	Test Solutions		**Log_{10} Reduction**
Adipose Sample Number	Control (Water Wash)	HOBr (1147 ppm as Br_2)	"Control vs. HOBr"[1]
1	6.15	4.27	1.88
2	5.96	4.76	1.20
3	6.42	4.37	2.05
4	6.00	4.32	1.68
5	6.98	5.36	1.62
6	7.18	4.20	2.98
7	6.11	4.58	1.53
8	6.55	4.03	2.52
9	6.08	5.01	1.07
10	7.06	5.05	2.01
Mean	**6.45**	**4.60**	**1.85**
S.D.[3]	**0.47**	**0.43**	**0.58**
C.V.[3]	**0.07**	**0.09**	**0.31**

t-Test: Two-Sample Assuming Equal Variances			
		"Control vs. HOBr" Samples	
		Variable 1	*Variable 2*
Mean		6.449	4.595
Variance		0.22021	0.188783333
Observations		10	10
Pooled Variance		0.204496667	
Hypothesized Mean Difference		0	
df		18	
t Stat[2]		9.167514706	
P(T<=t) one-tail		1.67199E-08	
t Critical one-tail		1.734063592	
P(T<=t) two-tail		3.34397E-08	
t value		2.100922037	

[1] "Control vs. HOBr" refers to log10 difference in APC (aerobic plate count) among the adipose pieces treated with Modesto city water compared to the HOBr solution at 1147 ppm as Br2

[2] Means within a grouping with an asterisk are significantly different (P<0.05) as determined by the t-test which assesses whether the means of two groups are statistically different from each other. Therefore if t Stat > t value the means of differences are significantly different. A probability of p = 0.05 (95% probability of making a correct statement).

[3] S.D. = Standard Deviation, C.V. = Coefficient of Variation