4031 Alvis Court
Rocklin, Ca 95677
Tel: 877.464.6649
Fax: 916.632.2139
E-mail: info@mionix.com

Effect of $Safe_2O^{®}_{brand}$ RTE 01 Applied at 60°C to Turkey Roll Products Inoculated With Cold Adapted *Listeria monocytogenes*

Objective:

Determine whether a 30-second treatment with $Safe_2O^{®}_{brand}$ RTE 01 applied at 60ºC can effectively produce a post-lethality effect on product inoculated with cold-adapted *Listeria monocytogenes*.

Materials and Methods:

Cold Adaptation Procedure and Culturing of *Listeria monocytogenes*

1. Add 25 ml of sterile BHI broth to Erlenmeyer flask.
2. Inoculate BHI broth with 50 µl of *Listeria monocytogenes* culture.
3. Cover flask with sterilized gauze and secure in place using a rubber band.
4. Place flask in 10ºC incubator.
5. Subculture by inoculating a new flask containing BHI broth as described in #2 at weekly intervals.
6. Check the bacterial growth pattern for 1-2 months by sampling an aliquot of the culture every 1-2 days. Determine *Listeria monocytogenes* titer at each time interval by serially diluting the sample and plating.
7. Incubate plates at 37ºC for 48 hours and count colony-forming units per sample. When the growth pattern of all strains is established, the cold adapted strains are available for prescribed studies.

Meat Inoculation and Treatment

1. Five strains of cold-adapted *Listeria monocytogenes* were cultured separately in BHI broth overnight at 10ºC. Prior to use, cultures will be mixed in equal proportions based on titer. The mixture was diluted 1:1,000 with sterile saline to produce a suspension for inoculation onto meat pieces. Meat inoculation level was determined by removing an aliquot of the mixture, making a serial dilution and plating same onto Tryptose Soy Agar (TSA) plates.
2. Oil Brown and Honey Roast Turkey Roll products containing 2.5% Opti-Form® were obtained from a manufacturer. Upon receipt, turkey rolls were carefully removed from the original packaging to a sterile surface in a laminar flow bio-safety hood. Thirty-six pieces were excised from the apical side of each type of turkey roll. Pieces measured approximately 1.2" X 1.2" X 0.3".
3. All pieces were irradiated with UV light for 30 minutes, and then inoculated with 20 micro liters of the *Listeria monocytogenes* suspensions onto the exterior side of each turkey meat piece. All inoculated pieces were kept in the laminar hood for an additional 90 minutes at ambient temperature to allow for bacteria attachment.
4. Inoculated Oil Brown and Honey Roast pieces were evenly divided into 3 groups (T, C_1 & C_2). Group T pieces were treated by submersion in 60ºC $Safe_2O^{®}_{brand}$ RTE 01 diluted 1:3 for 30 seconds. Group C_2 pieces were treated by submersion in 60ºC sterile deionized water for 30 seconds. After treatment, excess solution was allowed to drip off for 15 seconds and all pieces were then individually vacuum-packed and sealed.
5. Group C_1 pieces were directly transferred and sealed in vacuum bags without treatment.
6. All turkey pieces were incubated at 4ºC prior to removal for Listeria determination. Listeria determinations were carried out at one hour post-treatment and at 24 hours and at 20 and 40 days post-treatment.
7. For each set time period, three bags from each group were unpacked and 5 ml of sterile 0.1% peptone water was added to each bag. *Listeria monocytogenes* organisms were washed off the surface of each turkey piece by a two-minute hand massage of the bag. The number of *Listeria monocytogenes* colony-forming units (CFU) per turkey piece was determined by serial dilution of an aliquot from each rinsate followed by plating on Modified Oxford Selective Agar plates. All plates were incubated at 37ºC for 40-48 hours before CFU determination.

Results:

The Oil Brown and Honey Roast turkey roll products used for this study contained an outgrowth inhibitor, i.e., Purasal *Opti-Form*™, added at a 2.5% concentration. The turkey roll products, therefore, under the criterion specified in F.S.I.S. Directive 10,240.4, can be categorized "Alternative 2". The objective of the studies presented herein was to determine whether a 30-second treatment with $Safe_2O^{®}_{brand}$ RTE 01 applied at 60ºC can effectively produce a post-lethality effect on product inoculated with cold adapted *Listeria monocytogenes*.

Table 1: Effect of a 30-second treatment with $Safe_2O^{®}_{brand}$ RTE 01 applied at to <u>Oil Brown</u> Turkey inoculated with *Listeria monocytogenes.*

Time (Hours)	Treatment	CFU/piece	Ā of CFU/piece	Log Value	Log Reduction from Water Treatment	Log Reduction from No Treatment
1	No Treatment	3.37E+04	3.72E+04	4.57		
		3.73E+04				
		4.07E+04				
	Sterile deionized H_2O 60°C X 30" Dip	8.07E+03	7.67E+03	3.88		
		1.08E+04				
		4.13E+03				
	RTE-01 1:3 60°C X 30" Dip	2.44E+03	2.05E+03	3.31	0.57	1.26
		1.62E+03				
		2.10E+03				
24	No Treatment	2.67E+04	3.21E+04	4.51		
		3.47E+04				
		3.50E+04				
	Sterile deionized H_2O 60°C X 30" Dip	9.67E+02	1.08E+04	4.03		
		1.49E+04				
		1.65E+04				
	RTE 01 1:3 60°C X 30" Dip	2.10E+03	1.28E+03	3.11	0.93	1.40
		1.60E+03				
		1.30E+02				

Table 1 (Continued): Effect of a 30-second treatment with Safe$_2$O$^{®}_{brand}$ RTE 01 applied at 60°C to <u>Oil Brown</u> Turkey inoculated with *Listeria monocytogenes.*

Time (Days)	Treatment	CFU/piece	Ā of CFU/piece	Log Value	Log Reduction from Water Treatment	Log Reduction from No Treatment
20	No Treatment	1.12E+06 5.30E+05 3.90E+05	6.80E+05	5.83		
	Sterile deionized H_2O 60°C X 30" Dip	7.00E+06 5.23E+06 1.27E+06	7.67E+03	6.65		
	RTE-01 1:3 60°C X 30" Dip	5.15E+02 3.80E+02 2.87E+03	6.80E+05	3.10	3.55	2.73
40	No Treatment	4.90E+06 1.56E+06 2.13E+06	2.86E+06	6.46		
	Sterile deionized H_2O 60°C X 30" Dip	> 5.00E+07 2.88E+07 5.00E+07	> 4.29E+07	> 7.63		
	RTE 01 1:3 60°C X 30" Dip	6.80E+02 4.93E+03 1.27E+03	2.29E+03	3.36	4.27	3.10

In preliminary studies, taste panel tests showed application of a 1:3 dilution of Safe$_2$O$^{®}_{brand}$ RTE 01 diluted 1:3 on turkey roll products by a deluge or submersion process had no impact on organoleptic properties. In addition to organoleptic evaluations, it was determined that treatment with Safe$_2$O$^{®}_{brand}$ RTE 01 diluted 1:3 at temperatures ranging from 10-40ºC and at times ranging from 5-20 seconds did not functionally bring about a post-lethality effect of at least 1 log. Therefore, the studies described below were designed to determine if treatment with Safe$_2$O$^{®}_{brand}$ RTE 01 diluted 1:3 when applied at 60°C for 30 seconds could bring about a post-lethality effect of at least 1 log.

Studies in duplicate were carried out to access the post-lethality effect of a 30-second treatment with Safe$_2$O$^{®}_{brand}$ RTE 01 when applied at 60°C for 30 seconds. For Study #1, all turkey pieces excised from the apical side of Oil Brown and Honey Roasted Turkey rolls were inoculated with 5.53 X 10^4

CFU of a five-strain suspension of *Listeria monocytogenes* per piece (see Tables 1 and 2). For Study #2, all pieces were inoculated 4.43×10^4 CFU of a five-strain suspension of *Listeria monocytogenes* per piece (see Tables 3 and 4).

Study #1

As can be seen from Table 1, water treatment alone applied for 30 seconds at 60°C causes a post-lethality effect of 0.69 logs, relative to the initial inoculation level, determined at one hour post-treatment. The reduction is not enough to qualify as a post-lethality treatment under the criterion specified in F.S.I.S. Directive 10,240.4. However, when the Oil Brown product was treated for 30 seconds at 60°C with $Safe_2O^{®}_{brand}$ RTE 01 diluted 1:3, a reduction of 1.26 logs was observed.

Table 2: Effect of a 30-second treatment with $Safe_2O^{®}_{brand}$ RTE 01 applied at 60°C to Honey Roast turkey inoculated with *Listeria monocytogenes.*

Time (Hours)	Treatment	CFU/piece	Ā of CFU/piece	Log Value	Log Reduction from Water Treatment	Log Reduction from No Treatment
1	No Treatment	3.85E+04	3.85E+04	4.59		
		3.97E+04				
		4.17E+04				
	Sterile deionized H_2O 60°C X 30" Dip	8.10E+03	8.02E+03	3.90		
		6.80E+03				
		9.17E+03				
	RTE-01 1:3 60°C X 30" Dip	2.00E+01	< 1.70E+02	2.23	> 1.67	> 2.35
		< 5.00E+00				
		4.85E+02				
24	No Treatment	2.70E+04	3.03E+04	4.48		
		2.70E+04				
		3.70E+04				
	Sterile deionized H_2O 60°C X 30" Dip	5.70E+03	1.09E+04	4.04		
		1.33E+04				
		1.38E+04				
	RTE-01 1:3 4°C X 30" Dip	8.00E+01	< 3.00E+01	1.48	> 2.56	> 3.00
		< 5.00E+00				
		< 5.00E+00				

Table 2 (Continued): Effect of a 30-second treatment with $Safe_2O^{®}_{brand}$ RTE 01 applied at 60°C to <u>Honey Roast</u> Turkey inoculated with *Listeria monocytogenes.*

Time (Days)	Treatment	CFU/piece	Ā of CFU/piece	Log Value	Log Reduction from Water Treatment	Log Reduction from No Treatment
20	No Treatment	1.00E+05 5.00E+04 5.67E+04	6.89E+04	4.84		
	Sterile deionized H2O 60°C X 30" Dip	4.70E+05 8.27E+05 5.97E+05	6.31E+05	5.80		
	RTE-01 1:3 60°C X 30" Dip	< 6.50E+01 < 5.00E+00 < 5.00E+00	< 2.50E+01	< 1.40	> 4.40	> 3.44
40	No Treatment	2.15E+05 2.73E+06 3.17E+06	2.04E+06	6.31		
	Sterile deionized H_2O 60°C X 30" Dip	3.11E+07 1.36E+06 > 1.00E+08	> 4.42E+07	> 7.64		
	RTE 01 1:3 60°C X 30" Dip	< 5.00E+00 < 5.00E+00 < 5.00E+00	< 5.00E+00	< 0.70	> 6.95	> 5.61

Previous studies suggested the post-lethality effect should be greater. However, the apical surface of the tested Oil Brown turkey product has a very rough surface consisting of crunchy brown turkey. In addition, the surface is hydrophobic by nature, making it very difficult for water based, i.e., a hydrophilic antimicrobial to contact and eliminate bacteria on the surface of the product. Nevertheless, because $Safe_2O^{®}_{brand}$ RTE 01 has residual activity, it was deemed prudent to assess the post-lethality effect at 24 hours post-treatment. As can be seen from Table 1, the post-lethality effect increased from 1.29 logs to 1.40 logs at 24 hours post-treatment.

Having noted the post-lethality effect on Oil Brown turkey, similar studies were performed on a Honey Roast turkey product. A protocol identical to that for Oil Brown turkey was used and the observed

reduction of water treatment alone was 0.69 logs at one hour post-treatment (Table 2), i.e., the same as that shown for Oil Brown turkey (Table 1). However, the post-lethality effect noted at one hour post-treatment for Honey Roast turkey was >2.35 logs and the post-lethality effect observed at 24 hours was >3.00 logs.

From the studies described above, it was evident that a treatment at 60°C with $Safe_2O^{®}_{brand}$ RTE 01 diluted 1:3 for 30 seconds is effective in bringing about a post-lethality effect of >1 log by one hour post-treatment. Still to be resolved was the effect of treatment on shelf-life of the products with respect to Listeria replication.

Table 3: Effect of a 30-second treatment with $Safe_2O^{®}_{brand}$ RTE 01 applied at 60°C to <u>Oil Brown</u> Turkey inoculated with *Listeria monocytogenes.*

Time (Hours)	Treatment	CFU/piece	Ā of CFU/piece	Log Value	Log Reduction from Water Treatment	Log Reduction from No Treatment
1	No Treatment	4.10E+04 2.67E+04 3.23E+04	3.33E+04	4.52		
	DH_2O 60°C X 30" Dip	1.06E+04 1.90E+04 1.66E+04	1.54E+04	4.19		
	RTE01 1:3 60°C X 30" Dip	6.15E+02 9.20E+02 2.40E+03	1.31E+03	3.12	1.07	1.41
24	No Treatment	3.07E+04 2.63E+04 3.67E+04	3.12E+04	4.49		
	DH_2O 60°C X 30" Dip	6.37E+03 1.04E+04 6.67E+02	7.81E+03	3.89		
	RTE01 1:3 60°C X 30" Dip	1.09E+03 2.60E+03 1.70E+02	1.29+03	3.11	0.78	1.39

Table 3 (Continued): Effect on outgrowth of a 30-second treatment with $Safe_2O^{®}_{brand}$ RTE 01 applied at 60°C to Oil Brown Turkey containing inoculated with *Listeria monocytogenes.*

Time (Days)	Treatment	CFU/piece	Ā of CFU/piece	Log Value	Log Reduction from Water Treatment	Log Reduction from No Treatment
20	No Treatment	2.67E+04 6.67E+03 2.67E+04	2.00E+04	4.30		
	Sterile Deionized H2O 60°C X 30" Dip	3.33E+03 1.17E+03 1.17E+03	1.89E+03	3.28		
	RTE-01 1:3 60°C X 30" Dip	4.25E+02 2.60E+02 1.34E+03	6.75E+02	2.83	0.45	1.47
40	No Treatment	2.90E+04 2.70E+04 2.43E+04	2.68E+04	4.43		
	Sterile deionized H_2O 60°C X 30" Dip	1.33E+04 2.67E+04 2.00E+04	2.00E+04	4.30		
	RTE 01 1:3 60°C X 30" Dip	1.57E+03 2.71E+03 ND	2.14E+03	3.33	0.97	1.10

To assess the effect of treatment on shelf-life , turkey pieces were incubated at 4°C for 20 and 40 days, respectively, from inoculation with microbial assays being carried out at the defined times. As can be seen from Table 1, at 20 and 40 days post-inoculation, there was notable outgrowth of Listeria on the Oil Brown turkey, even though a grow out inhibitor was added, i.e., the observed Listeria CFU/piece increased by 1.27 and 1.89 logs, respectively, over that of the initial inoculation levels of 4.57 logs. In contrast, the levels of Listeria associated with Oil Brown turkey treated for 30 seconds at 60°C with $Safe_2O^{®}_{brand}$ RTE 01 diluted 1:3 was decreased by 1.47 and 1.21 logs, respectively, at 20 and 40 days post-treatment.

Similar results were noted for Honey Roast turkey Table 2). Specifically, at 20 days the detectable level of Listeria/piece decreased by 0.25 logs, but by 40 days post-inoculation the level had increased by 1.72 logs over the initial inoculation level of 4.57 logs. In contrast, at 20 and 40 days post-inoculation the level of Listeria/piece for Honey Roast product treated for 30 seconds at 60°C with $Safe_2O^{®}_{brand}$ RTE 01 diluted 1:3 decreased by >1.13 and 1.04 logs, respectively (Table 2). The studies described above were repeated to further evaluate and confirm the effects of a 30-second treatment at 60°C with $Safe_2O^{®}_{brand}$ RTE 01 diluted 1:3. As observed for this study, there was, in effect, no outgrowth on the Oil Brown control turkey product (Table 3). The post-lethality effect observed at one hour post-treatment was 1.41 logs, slightly greater than that observed in Study #1. There was no outgrowth observed over the 40-day study period.

Table 4: Post-lethality effect of a 30-second treatment with $Safe_2O^{®}_{brand}$ RTE 01 applied at 60°C to Honey Roast Turkey inoculated with *Listeria monocytogenes.*

Time (Hours)	Treatment	CFU/piece	Ā of CFU/piece	Log Value	Log Reduction from No Treatment	Log Reduction from Non Treated
1	No Treatment	3.13E+04 3.37E+04 2.70E+04	3.07E+04	4.49		
	Sterile deionized H_2O 60°C X 30" Dip	8.73E+03 9.73E+03 5.70E+03	8.05E+03	3.91		
	RTE01 1:3 60°C X 30" Dip	5.00E+00 <5.00E+00 <5.00E+00	< 5.00E+00	<0.70	> 3.21	> 3.79
24	Without Any Treatment	3.60E+04 3.47E+04 3.30E+04	3.46E+04	4.54		
	Sterile deionized H_2O 60°C X 30" Dip	1.12E+04 1.56E+04 8.77E+03	1.19E+04	4.07		
	RTE01 1:3 60°C X 30" Dip	2.15E+02 <5.00E+00 <5.00E+00	< 7.50E+01	< 1.88	> 2.20	> 2.66

Table 4 (Continued): Effect on outgrowth of a 30-second treatment with $Safe_2O^{®}_{brand}$ RTE 01 applied at 60°C to Oil Brown Turkey containing inoculated with *Listeria monocytogenes.*

Time (Days)	Treatment	CFU/piece	Ā of CFU/piece	Log Value	Log Reduction from Water Treatment	Log Reduction from No Treatment
20	No Treatment	4.50E+04 6.60E+03 4.17E+04	5.09E+04	4.71		
	Sterile deionized H2O 60°C X 30" Dip	1.73E+03 3.67E+03 6.63E+03	9.10E+04	4.96		
	RTE-01 1:3 60°C X 30" Dip	3.50E+02 < 5.00E+02 < 5.00E+03	< 1.50E+01	< 1.18	> 3.78	> 3.53
40	No Treatment	3.20E+04 3.30E+04 3.70E+04	3.40E+04	4.53		
	Sterile deionized H_2O 60°C X 30" Dip	8.33E+04 3.10E+04 1.40E+04	1.78+05	5.25		
	RTE 01 1:3 60°C X 30" Dip	0.00E+00* 0.00E+00* 0.00E+00*	0.00E+00		5.25	4.53

*Zero CFU/turkey piece verified by enrichment

Again, as observed for the Oil Brown turkey control (Table 3), there was little or no outgrowth detected for the Honey Roast turkey control (Table 4), in this study. However, the post-lethality effect observed at one hour post-treatment was increased by ~ 1 log. In addition, no CFU were detected at 40 days post-treatment with $Safe_2O^{®}_{brand}$ RTE 01 diluted 1:3 at 60°C for 30 seconds. This suggested there were no viable colonies associated with the Honey Roasted turkey pieces at this point. To demonstrate this was, in fact, the case, meat pieces were placed in enrichment medium and incubated for 24 hours. An aliquot from the enrichment media was plated to determine if viable Listeria colonies could be detected. Zero Listeria CFU were detected.

Conclusions:

Under the criterion specified in F.S.I.S. Directive 10,240.4, in order for product to be categorized as "Alternative 1," a product must be subjected to a post-lethality treatment that brings about at least a 1 log reduction and outgrowth must not exceed 2 logs. In every case as shown herein, there was a post-lethality effect meeting the criterion as specified in Directive 10240.4 when Oil Brown and Honey Roasted turkey products were treated with $Safe_2O^{®}_{brand}$ RTE 01 diluted 1:3 for 30 seconds (see Tables 1-4).

The studies described herein were carried out using cold-adapted Listeria for a specific reason. Data not yet published suggests the lactate/diacetate combination may not prevent outgrowth of cold-adapted *Listeria monocytogenes* strains, such as those that may be found in a meat production plant. Therefore, meat so treated would be classified as "Alternative 3" if this was the only additive or process used for Listeria control. Based on the studies presented herein, it is difficult to determine the impact of using cold-adapted Listeria strains for validation studies. However, it is obvious that Listeria that may be growing in meat production plants are more likely adapted to grow at lower temperatures. Many Listeria challenge studies use Listeria strains cultured at ambient or higher laboratory conditions, i.e., temperatures often in excess of 25ºC. It is probable that they are more sensitive to antimicrobials because they have to adapt to conditions used in validation studies, i.e., temperatures lower than 8ºC. However, cold-adapted strains are fully competent to replicate under these same conditions, i.e., a validation study may be compromised by the adaptation process of bacteria cultured at elevated temperatures. In fact, it is noteworthy that there is considerable outgrowth on the Oil Brown turkey control product (Table 1). The lactate/diacetate additive Purasal *Opti-Form*™ was incorporated into this product at a concentration of 2.5% for the purpose of preventing outgrowth. Nevertheless, for the studies presented in Table 1, elevated levels of Listeria were detected such as to question the functional capacity of the outgrowth inhibitor in the face of a challenge with cold-adapted Listeria.

It is important to note that an aqueous solution coming into contact with the surface of a meat product in which lactate/diacetate has been added, including the Oil Brown or Honey Roasted products used for this study, seems to cause a reduction in concentration of the surface levels of the outgrowth inhibitor, thereby allowing Listeria to grow beyond 2 logs. It is also quite possible that a similar physical effect may occur during heat pasteurization. Heat pasteurization causes a certain amount of water to be effectively purged. Purge formation could, in effect, mimic the solubilization effect of an aqueous application and is an effect that needs to be considered in the processing of ready-to-eat meat products. In consideration of this effect, treatment with $Safe_2O^{®}_{brand}$ RTE 01 diluted 1:3 for 30 seconds completely ablated any potential problem in this regard.