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FINAL REPORT
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VALIDATION STUDY

Evaluation of $Safe_2O^{®}_{brand}$RTE 01 as an Antimicrobial Agent and Post-Lethality Treatment for Classification of Turkey Products into USDA-FSIS Directive 10,240.4 Alternative 1

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STUDY SPONSOR

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MORONI FEED CO.
"Progress Through Cooperation"

OBJECTIVE

The overall objective of this study was to determine whether $Safe_2O^{®}_{brand}$RTE 01 qualifies as both an antimicrobial agent and post-lethality treatment for classification of turkey products into USDA-FSIS Directive 10,240.4 Alternative 1.

INTRODUCTION

Classification of a ready-to-eat product into USDA-FSIS Directive 10,240.4 Alternative 1 requires a post-lethality treatment to reduce or eliminate *Listeria monocytogenes* and an antimicrobial agent or process to limit growth of the pathogen over the shelf-life of the RTE product. A post-lethality treatment, which may also be an antimicrobial agent or process, will ideally reduce *L. monocytogenes* by ≥ 2.0 $\log_{10}$, but may also be between 1.0 – 2.0 $\log_{10}$ with the associated possibility of more sampling by FSIS. A treatment that reduces *L. monocytogenes* by less than 1.0 $\log_{10}$ is not eligible as a post-lethality treatment. An antimicrobial agent or process will ideally limit the outgrowth of *L. monocytogenes* to ≤ 1.0 $\log_{10}$ over the course of the product shelf-life. The ability to control outgrowth to > 1.0 $\log_{10}$ but less than 2.0 $\log_{10}$ would still qualify an agent or process as antimicrobial, but with an associated increased level of sampling by FSIS. Outgrowth of > 2.0 $\log_{10}$ during a product shelf-life period would disqualify an agent or process as antimicrobial in accordance with Directive 10,240.4. The overall objective of this study is to determine if $Safe_2O^{®}_{brand}$RTE 01 qualifies as both a post-lethality treatment and antimicrobial agent based on the guidelines provided in Directive 10,240.4.

MATERIALS AND METHODS

Preparation of Inoculum

To determine whether the $Safe_2O^{®}_{brand}$RTE 01 treatment supports classification of the test turkey products evaluated into Alternative 1, the following cultures were prepared:

- *Listeria monocytogenes* (ATCC 19114)
- *Listeria monocytogenes* (ATCC 19115)
- *Listeria monocytogenes* (ATCC 13932)
- *Listeria innocua* (ATCC 33090)
- *Listeria monocytogenes* (Food Safety Net Services environmental sample – FSNS 1000)

Fresh cultures of each organism were prepared from frozen stock cultures by growing separately in Tryptic Soy Broth (TSB – Difco 211822; Becton Dickenson, Sparks, MD) containing 0.6% yeast extract (YE – Difco 212750; BD, Sparks, MD) at 35°C for 24 hours. The cultures were passed through TSB-YE three times, with each pass verified for purity and performance by plating onto Sheep Blood Agar, Trypticase Soy Agar (TSA – Difco 236950; BD, Sparks, MD), and Modified Oxford Agar (MOX - Base: Difco 222530, Modified Oxford Antimicrobic Supplement: Difco 211763; BD, Sparks, MD). After passing through TSB-YE, each culture, upon reaching its optimal growth phase, was inoculated into fresh TSB-YE and incubated at 25°C for 18 hours, then held at 4°C for 10 hours. This procedure supports adaptation of the cultures to growth under refrigerated conditions. The liquid cultures were then combined into a cocktail, centrifuged, washed, and resuspended in fresh TSB. The resulting cocktail was diluted to an approximate concentration target of 1 x 10^4 colony forming units (CFU)/ml of *Listeria*. The diluted cocktail was used to inoculate the samples evaluated in this study as described below.

Inoculation, Treatment and Enumeration

Products received for this study were packaged and supplied as normal for commerce. Product examined in this study were as follows: 1) Cured, Smoked Deli (60364) – 9 pound deli with a 5" profile height; and, 2) Uncured, Pan Roasted/Pre-Brown Deli (60142) – 8 pound deli with a 4" profile height.

Each product was aseptically handled and sub-sectioned into 1.2" x 1.2" x 0.3" slices for inoculation, treatment and repackaging for storage at 38 - 40°F. Slices were collected from the product surface of each test product based on the higher risk of surface association of *Listeria* contamination vs. internal contamination. Each product slice was surface inoculated using a sterile pipette. A volume of the microbial cocktail of the 5 strains of *Listeria* spp. described above was spread across the surface of the sub-section to deliver approximately 1 X 10^4 CFU of pathogen per slice. The weight of each slice was noted such that the microbial counts per unit weight can be calculated upon enumeration. Each product was inoculated to provide triplicate treated and untreated controls for each test point. The inoculated products were held at 38 - 40°F for 30 minutes to support attachment of *Listeria* to the product surface. In addition, triplicate samples for each product type were assessed for the presence of background *Listeria* through direct processing and enumeration of uninoculated controls.

Treatment of each inoculated product with $Safe_2O^{®}_{brand}$RTE 01 followed a submersion procedure based on previous studies.[1] A submersion treatment of test products with $Safe_2O^{®}_{brand}$RTE 01 has been historically used in laboratory

trials for validation purposes based on the complete surface coverage that it offers which is similar to the product surface saturation that occurs in the processing plant environment using a large volume of spray at low pressure and/or actual product dipping. An additional set of untreated products was maintained at 38 - 40°F during the applicable time period as treated products undergo the treatment process for comparison of *Listeria* outgrowth levels between treated and untreated samples.

The ability of $Safe_2O^{®}_{brand}$RTE 01 to classify as a post-lethality treatment was first examined by treating products as follows and enumerating *Listeria* after 1 hr hours of storage at 38 - 40°F for treated and untreated controls. Triplicate samples were fully submerged in 60°C $Safe_2O^{®}_{brand}$RTE 01 diluted 1:3 in sterile, deionized water for 30 seconds. After exposure, the product was aseptically removed, allowing residual $Safe_2O^{®}_{brand}$RTE 01 to drip from the surface for 15 seconds, and was vacuum packaged for storage at 38 - 40°F. *Listeria* was enumerated as described below for triplicate treated and untreated controls. If greater than 1 $\log_{10}$ reduction was achieved, additional samples were treated to monitor antimicrobial activity of $Safe_2O^{®}_{brand}$RTE 01 and ability to control outgrowth of *Listeria* to less than 2.0 $\log_{10}$ over the shelf-life of each product on the following designated test days: 1) Cured, Smoked Deli (60364) – Days 30, 45, 60 and 75; and, 2) Uncured, Pan Roasted/Pre-Brown Deli (60142) – Days 30, 45 and 60.

Enumeration of *Listeria* was performed by aseptically opening each package, transferring the product to a sterile, Whirl-Pak™ bag and adding sterile, Butterfield's phosphate buffer (BPB) as an equal volume to weight based on the original sample weight. At least 5 ml of the BPB will be transferred back to the original packaging using a sterile pipette, to "rinse" the interior surface and collect any remaining *Listeria* which will be transferred to the same Whirl-Pak™ bag containing the corresponding product sample. The Whirl-Pak™ bag containing the product sample and package rinsate was placed in a Stomacher® for 2 minutes on medium to high speed. All samples were plated onto Modified Oxford Agar (MOX – Base: Difco 222530, Modified Oxford Antimicrobic Supplement: Difco 211763; Becton Dickinson, Sparks, MD) at appropriate dilutions using a spiral plater (Spiral Biotech Autoplate 4000) to obtain plates within the countable range. MOX Plates were incubated for 24 hours at 35°C, and total *Listeria* counts were enumerated on an automated counting system (Advanced Instruments 510 using Q Count™ software, Version 1.5). Total *Listeria* for each sample was averaged and the corresponding $\log_{10}$ values were determined. Reduction and/or outgrowth of *Listeria monocytogenes* was calculated for each test point and was compared to an inoculated, untreated control.

RESULTS AND DISCUSSION

Data for the Day 0, 1 hour samples is shown in Table 1 below, including type of sample, actual raw counts (in CFU/g) for each replicate, average count (in CFU/g) for all three replicates, the $\log_{10}$ of the average, and, in the case of the Treated samples, the reduction (in $\log_{10}$ CFU/g) seen from the Untreated samples.

Table 1. Day 0, 1 Hour sample Data

Sample Type	Replicate (CFU/g) 1	2	3	Average (CFU/g)	Log_{10}	Reduction
Uncured Pan-Roasted Uninoculated	<10	<10	<10	<10	n/a[1]	n/a
Cured Smoked Uninoculated	<10	<10	<10	<10	n/a	n/a
Uncured Pan-Roasted Untreated	3,370	3,270	5,520	4,053	3.61	n/a
Cured Smoked Untreated	5,520	10,700	2,960	6,393	3.81	n/a
Uncured Pan-Roasted Treated	102	102	204	136	2.13	1.47
Cured Smoked Treated	409	204	102	238	2.38	1.43

[1]n/a = Not applicable

Listeria was reduced greater than 1.4 logs in both uncured pan-roasted and cured smoked samples after one hour of incubation. No background *Listeria* was detected in the uninoculated samples.

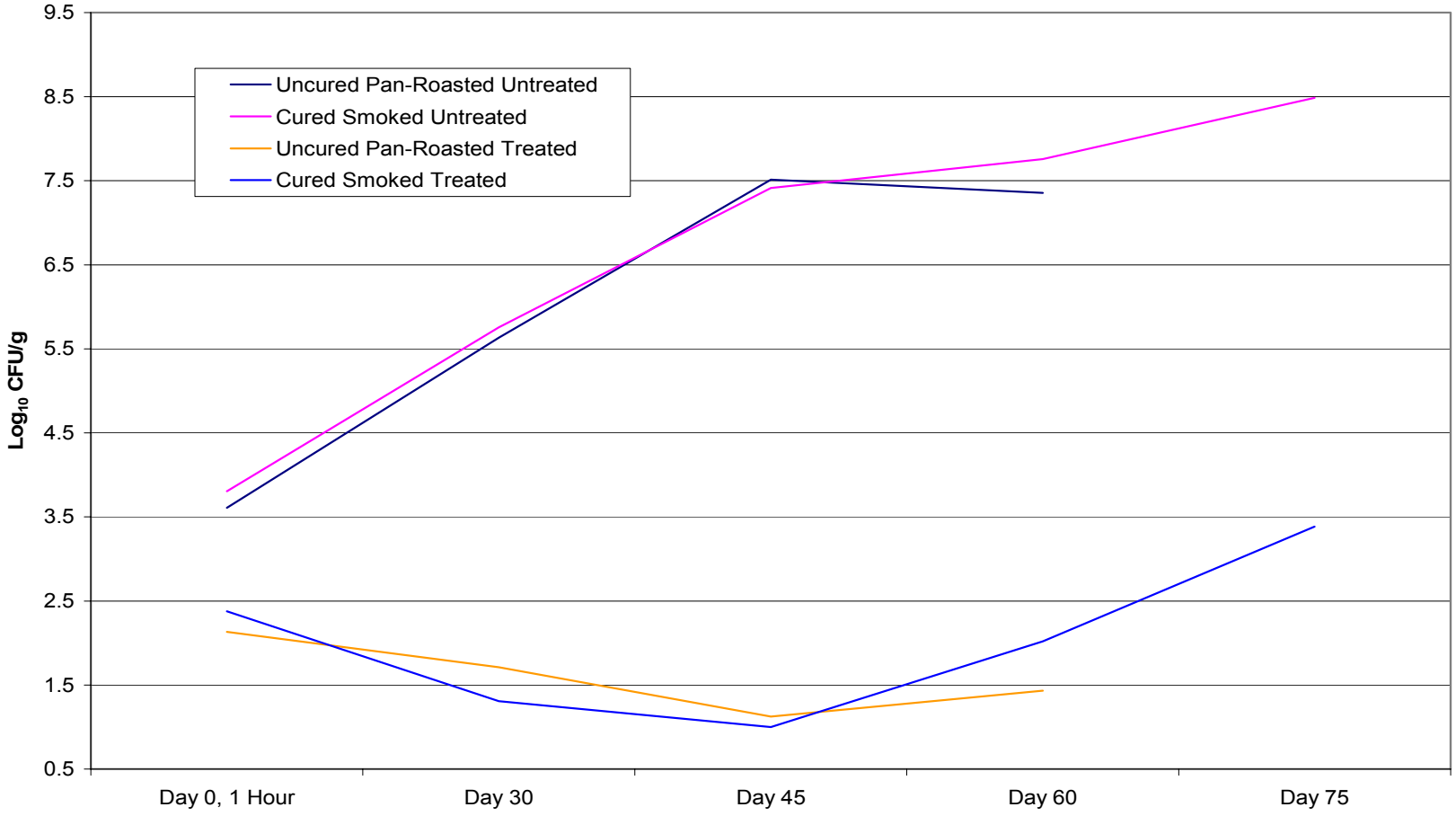
Data for the remaining shelf life samples is shown in Table 2 below, including type of sample, actual raw counts (in CFU/g) for each replicate, average count (in CFU/g) for all three replicates, the $\log_{10}$ of the average, and the outgrowth or reduction of *Listeria*, as compared to level of *Listeria* observed at Day 0. If the amount of *Listeria* has outgrown beyond the initial inoculum, Outgrowth/Reduction is shown as a positive number. If the amount of *Listeria* has been reduced below the initial inoculum, Outgrowth/Reduction is shown as a negative number. Data is also displayed graphically in Figure 1.

Table 2. Raw Shelf Life Data

Day 30	**Replicate (CFU/g)**			**Average**		**Outgrowth/**
Sample Type	**1**	**2**	**3**	**(CFU/g)**	**Log_{10}**	**Reduction**
Uncured Pan-Roasted Untreated	316,000	276,000	695,000	429,000	5.63	2.02
Cured Smoked Untreated	633,000	411,000	660,000	568,000	5.75	1.95
Uncured Pan-Roasted Treated	72	10	72	51	1.71	-0.42
Cured Smoked Treated	41	10	10	20	1.31	-1.07
Day 45	**Replicate (CFU/g)**			**Average**		**Outgrowth/**
Sample Type	**1**	**2**	**3**	**(CFU/g)**	**Log_{10}**	**Reduction**
Uncured Pan-Roasted Untreated	33,000,000	37,300,000	27,400,000	32,566,667	7.51	3.90
Cured Smoked Untreated	15,900,000	52,000,000	10,200,000	26,033,333	7.42	3.61
Uncured Pan-Roasted Treated	10	20	10	13	1.12	-1.01
Cured Smoked Treated	10	10	10	10	1.00	-1.38
Day 60	**Replicate (CFU/g)**			**Average**		**Outgrowth/**
Sample Type	**1**	**2**	**3**	**(CFU/g)**	**Log_{10}**	**Reduction**
Uncured Pan-Roasted Untreated	16,400,000	28,500,000	23,300,000	22,733,333	7.36	3.75
Cured Smoked Untreated	55,200,000	38,800,000	77,700,000	57,233,333	7.76	3.95
Uncured Pan-Roasted Treated	10	61	10	27	1.43	-0.70
Cured Smoked Treated	102	102	110	105	2.02	-0.36
Day 75	**Replicate (CFU/g)**			**Average**		**Outgrowth/**
Sample Type	**1**	**2**	**3**	**(CFU/g)**	**Log_{10}**	**Reduction**
Cured Smoked Untreated	409,000,000	259,000,000	248,000,000	305,333,333	8.48	4.68
Cured Smoked Treated	4,090	2,040	1,160	2,430	3.39	1.01

Listeria levels in untreated uncured pan-roasted and untreated cured smoked samples increased from the Day 0, 1 Hour samples to a final outgrowth amount of 3.75 logs for the uncured pan-roasted and 4.68 logs for the cured smoked treated. *Listeria* levels in the treated samples continued to drop until Day 45. Final outgrowth/reduction levels observed for the treated samples were -0.70 for the uncured pan-roasted and 1.01 for the cured smoked.

Figure 1. Growth of *Listeria* in Turkey products



CONCLUSIONS

The data in this study indicates that the $Safe_2O^{®}_{brand}$RTE 01 treatment is effective as both a post-lethality treatment and an antimicrobial treatment in accordance with USDA-FSIS Directive 10,240.4 for the turkey products examined at a shelf life of up to 60 days when vacuum packaged and stored at 38 - 40°F. The initial reductions of 1.47 and 1.43 for uncured pan-roasted turkey and cured smoked turkey fulfill the requirements of at least a 1.0 log initial reduction. $Safe_2O^{®}_{brand}$RTE 01 also qualifies as an antimicrobial treatment for uncured pan-roasted turkey at 60 days with an observed reduction of -0.70 logs. A *Listeria* outgrowth of 1.01 at 75 days for cured smoked turkey similarly qualifies as an antimicrobial treatment, although with increased monitoring. Therefore, at 60 days of shelf life, the $Safe_2O^{®}_{brand}$RTE 01 qualifies under Alternative 1 (post-lethality and antimicrobial) of Directive 10,240.4, but at 75 days, $Safe_2O^{®}_{brand}$RTE 01 qualifies as Alternative 1, but only with increased monitoring by USDA-FSIS.

REFERENCES

1. Mionix Corp. Applied Innovations, "Effect of $Safe_2O^{®}_{brand}$RTE 01 Applied at 60°C to Turkey Roll Products Inoculated with Cold Adapted *Listeria monocytogenes*."

2. USDA-FSIS Directive 10,240.4. "Verification Procedures for the Listeria monocytogenes Regulation and Microbial Sampling of Ready-to-Eat (RTE) Products for the FSIS Verification Testing Program". Accessed at www.**fsis**.**usda**.gov/OPPDE/rdad/**FSISDirective**s/10**240**.4/10**240**_4Int.pdf.

FINAL REPORT APPROVAL

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