Altria Client Services
I N T E R O F F I C E C O R R E S P O N D E N C E
Richmond, Virginia

Altria
Altria Client Services

Research, Development & Engineering

To: Scott Williams **Date: Aug. 22, 2017**[1]

From: John Kotrola

Subject: Report on Copenhagen Snuff Fine Cut[2]

Objective

To determine the viability of select pathogens in select finished product during storage at retail conditions. Selected product will be produced and canned as under normal operations. The product cans will be challenged with select pathogens and stored.

Test Materials:

Copenhagen® Snuff Fine Cut ((b) (4)

Test Plan:

MST Materials

The products were produced and canned under normal operating conditions. The required number of cans were collected and sent to the CRT Industrial Microbiology lab for finishing. The cans were inoculated and the cans closed and placed in the appropriate atmosphere to maintain the oven volatiles (OV). The study was conducted by Silliker Food Science Center, Merieux NutriSciences, 3600 Eagle Nest Drive, South Building, Crete, IL 60417

Sample Preparation and Storage Conditions

The following sample preparation and storage were consistent for each sample. The samples were inoculated with 3 bacteria species. Each bacteria culture was divided into 3 subcultures for triplicate inoculation. A total of 90 cans per trial were inoculated individually with a 10^9 Colony-Forming Units (CFUs) starting bacteria inoculum at an inoculum-to-product ratio of 1/100. An additional loss of up to 1 log was expected reducing the resulting inoculation down to

[1] (b) (4)

[2] Copenhagen® Fine Cut and variants thereof have been on the market since 1822. The candidate product subject to the MRTPA is the product for which FDA granted grandfathered status (Grandfather Number – GF1200194) on November 1, 2012. The test product used in this study was manufactured according to the specifications of the grandfathered version.

10^6 CFU/g. A control sample was prepared in triplicate in this same manner using sterile Butterfield's Buffer as inoculum. Each trial set, or subculture, of 30 cans were stored in an environmental chamber with temperature and relative humidity controls set at 22°C, 86%RH. A total of 120 cans per product were needed to prepare the samples and conduct the study.

Challenge Microorganisms

Product was inoculated with composite cultures prepared from 3 strains (Table 1*)* maintained in the Silliker Food Science Center Culture Collection (FSC-CC; Table 1). The rationale for selecting these three microorganisms is that they represent the majority of pathogens that are responsible for foodborne illness. Individual strains of *Salmonella* spp. and *E. coli* O157:H7 were prepared by transferring each strain to individual tubes of Trypticase Soy Broth (TSB) for 18-24 h at 35°C. *L. monocytogenes* strains were propagated in TSB plus 0.6% Yeast Extract (TSBYE and incubated for 18-24 h at 35°C. Verification of *E. coli* O157:H7, *Salmonella spp.*, and *L. monocytogenes* was conducted by streak plating onto Sorbitol MacConkey agar (SMAC), Xylose Lysine Desoxycholate Agar (XLD), and Modified Oxford Agar (MOX) plates, respectively, and incubating at 35°C for 24 h. Typical reactions on selective agar plates were considered confirmatory. Latex agglutination, serological testing and biochemical testing using VITEK were used for further confirmation for *E. coli* O157:H7, *Salmonella* spp. and *L. monocytogenes*, respectively.

Broth cultures of *Salmonella* and *E. coli* O157:H7 were then spread plated to form lawns onto at least 30 plates each of Tryptic Soy Agar (TSA) and incubated at 35°C for 24 h.

Due to natural microflora in the products that may also grow on MOX, making it difficult to distinguish from inoculated *L. monocytogenes* on the plate, *L. monocytogenes* cultures were induced to be resistant to 100 µg Rifampin/ml.

Broth cultures of *L. monocytogenes* were spread plated to form lawns onto at least 30 plates of Tryptic Soy Agar (TSA) plus 0.6% Yeast Extract supplemented with 100 µg Rifampin/ml and incubated at 35°C for 24 h. Following incubation, approximately 1 to 2 ml of phosphate buffer was added to each plate. The bacterial lawn was loosened with a sterile spreader and a sterile pipette was used to collect the cells. The cultures were centrifuged if necessary and re-suspended in phosphate buffer.

A cell suspension was prepared for each strain used in an inoculum. Cell suspensions were mixed to prepare an inoculum, which contained approximately equal numbers of cells of each strain. Composite bacterial cultures were stored at 4°C during enumeration by plate count methods.

Table 1. The List of Microorganisms

***Listeria monocytogenes* Composite**	**FSC-CC #**	**Source (Type)**
Listeria monocytogenes	501	Environmental meat (3b)
Listeria monocytogenes	2473	ATCC 49594, Scott A (4b)
Listeria monocytogenes	2492	ATCC 51772, Cheese (1/2a)
***Escherichia coli* O157:H7 Composite**	**FSC-CC #**	**Source (Toxin)**
Escherichia coli O157:H7	2841	ATCC 43895, Raw hamburger meat (*stx* 1 & 2)
Escherichia coli O157:H7	1430	ATCC 43889, Human feces with hemorrhagic uremic syndrome (*stx* 2)
Escherichia coli O157:H7	2842	ATCC 43890, Human feces (*stx* 1)
***Salmonella* spp. Composite**	**FSC-CC #**	**Source (Antigen)**
Salmonella Enteritidis	1395	ATCC 13076 (I 1,9,12:g,m)
Salmonella Senftenberg 775W	1249	ATCC 43845 (1, 3, 19:g,s,t: -)
Salmonella Typhimurium	1397	ATCC 13311 (I 4,5,12:i:1,2)

Preparation of Test Samples and Storage

Products were divided evenly and randomly for each challenge microorganism and an uninoculated control. Each can was inoculated with a composite culture at approximately 10^{8-9} (CFU) per gram. Three hundred microliter of inoculum ($10^{10\text{-}12}$ CFU/ml) were surfaced inoculated on the products within the can (for pouches – each pouch was individually inoculated with an equal amount of inoculum). Controls were inoculated with sterile Butterfields Phosphate Buffer. After inoculation, samples were collected as soon as possible and processed to determine the day 0 inoculation. The inoculated samples and control were stored at 21°C with 85%RH for the duration of the study.

Enumeration of the challenged and control products were on days 0, 2, 4, 7, 14, 21, 28, 35, 10 weeks and 20 weeks.

Methods of Analysis

Each product was analyzed for the presence/absence of *L. monocytogenes, Salmonella* and *E. coli* O157:H7 at the initiation of the study using the FDA BAM *(3)*.

For *E. coli* O157:H7, three 25 g samples were added into sterile stomacher bags containing 225 ml of Modified Buffered Peptone Water with Pyruvate (mBPWp) and incubated for 5 h at 37°C. Then 1 ml each of the ACV supplement (0.23% Acriflavin, 0.23% Cefsulodin, 0.18%

Vancomycin) was added into mBPWp and incubated at 42°C for 18-24h. The enrichment broth was streaked onto Tellurite Cefixime-Sorbitol MacConkey Agar (TC-SMAC) and incubated for 18-24 h at 37°C.

For *Salmonella* spp., three 25 g samples were added into sterile stomacher bags containing TSB. After 1 h at 35°C, each sample bag was adjusted to a neutral pH (6.8±0.2) and incubated for 24 h at 35°C. The preliminary enrichment was transferred to Rappaport-Vassilliadis (RV) and Tetrathionate (TT) broth and incubated for 24 h at 42°C and 35°C for RV and TT broth, respectively. The secondary enrichment broth was streaked onto XLD, Hektoen Enteric (HE) and Bismuth Sulfite (BS) agar and incubated at 35°C for 24 h for XLD and HE and 48 h for BS agar.

For *L. monocytogenes*, three 25 g samples were added into sterile stomacher bags containing Buffered *Listeria* Enrichment Broth (BLEB) with 2.5 ml of 10% sodium pyruvate. Sample bags were incubated at 30°C for 4 h and then selective agents (0.455 ml of 0.5% Acriflavin, 1.8 ml of 0.5% nalidixic acid and 1.15 ml of 1% cycloheximide) were added. Incubation was continued at 30°C for 24 h and 48 h. The enrichment broth from 24 h and 48 h was streaked onto MOX and incubated for 48 h at 35°C.

Samples of the control and inoculated portions were analyzed initially (i.e. after inoculation) and on days 2, 4, 7, 14, 21, 28, 35, 10 weeks and 20 weeks. Three analytical samples were taken at each interval and analyzed by plate count methods, providing a minimum of three results per data point *(1)*. The negative control and inoculated portions were analyzed by plate count methods for *L. monocytogenes*, *Salmonella* and *E. coli* O157:H7.

For negative control, 225 ml of phosphate buffer was added to 25 g of sample to make a 1:10 dilution. Remaining samples were used for water activity measurement. For inoculated portions, a 1:10 dilution of entire sample to phosphate buffer were "stomached" (macerated to allow preparation of uniform suspension) for 2 minutes and then serially diluted. The methods of analysis are listed in Table 2.

Table 2. Methods of Analysis

Test	Medium	Incubation Time/ Temperature/ Atmosphere
Listeria monocytogenes	Tryptic Soy Agar plus 0.6% Yeast Extract supplemented with 100 µg Rifampin/ml with Modified Oxford Agar overlay	48 h/30°C/aerobic
Salmonella	Trypticase Soy Agar with Xylose Lysine Desoxycholate overlay	48 h/35°C/aerobic
Escherichia coli O157:H7	Trypticase Soy Agar Sorbitol MacConkey Agar overlay	48 h/35°C/aerobic

Results and Discussion

Water activity levels of the samples ranged between 0.855 and 0.877 and did not change during storage at 21±1°C with 85±5% relative humidity for 20 weeks (Table 3). *L. monocytogenes*, *Salmonella* or *E. coli* O157:H7 were not detected from enrichment of non-inoculated samples at the initiation of the study. The levels of all three pathogens were less than the detection limit in non-inoculated samples in remaining sampling time points.

The average initial counts of *L. monocytogenes*, *E. coli* O157:H7 and *Salmonella* in the products ranged 8.60 – 8.65, 8.86 – 8.98, 9.04 – 9.08 Log_{10} CFU/g, respectively, while calculated inoculums levels were 8.41, 9.20 and 9.32 Log_{10} CFU/g, respectively (Table 3). The counts of *E. coli* O157:H7 and *Salmonella spp.* pathogens were below the detection limits after 7 days and 4 days respectively. Counts of *L. monocytogenes, which* remained detectable until day 21. By day 21 *L. monocytogenes*, level in the product had achieved an 8.63log reduction, which surpasses the acceptable reduction of 5 logs.

The difference in reductions between the 3 pathogens might be attributed to the differences in cell surface structures between *E. coli* O157:H7/*Salmonella* and *L. monocytogenes*. Plants protect themselves against pathogens by producing antimicrobial compounds or by activating host defenses. According to Seo and Mathews *(4),* bacterial surface components of *E. coli* O157:H7 played an important role in inducing plant defense response and contribute to the decreased numbers of the *E. coli* O157:H7. Similarly the levels of *E. coli* O157:H7 and *Salmonella* decreased drastically in the MST products within the current study compared to *Listeria*.

Overall, the Copenhagen Snuff Fine Cut product evaluated in this study demonstrated greater than 5 log reduction of *E. coli* O157:H7, *Salmonella* and *L. monocytogenes* within 21 days when stored at 21±1°C with 85±5% relative humidity and maintained that reduction through to the end of expected shelf life.

References

1. NACMCF Executive Secretariat. 2010. Parameters for determining inoculated pack/challenge study protocols. J. Food Prot. 73(1): 140-202.
2. Institute of Food Technologists. 2001. Evaluation and definition of potentially hazardous foods. (IFT/FDA Contract No. 223-98-2333. Task Order No.4. December 31.) Comp. Rev. in Food Sci. and Food Safety Vol 2. Supplement
3. FDA. Bacteriological Analytical Manual. 2011. http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm2006949.htm
4. Seo, S. and Matthews, K.R. 2012. Influence of the plant defense response to *Escherichia coli* O157:H7 cell surface structures on survival of that enteric pathogen on plant surfaces. Appl. Environ. Microbiol. 78(16): 5882-5889.

Table 3. Measurement of Water Activity and Enumeration of Microbial Pathogen Species Inoculum and Recovery from Challenged Copenhagen® Snuff Fine Cut

Table 3. Measurement of water activity and enumeration of microbial pathogen species inoculum and recovery from challenged Copenhagen Snuff Fine Cut

			Enumeration (Log_{10} CFU/mL)		
Sampling Date (Study Day)	**Sample**	**Water Activity**	***Salmonella. spp.***	***L. monocytogenes***	***E. coli***

(b) (4)

(b) (4)

			Enumeration (Log_{10} CFU/mL)		
Sampling Date (Study Day)	**Sample**	**Water Activity**	***Salmonella. spp.***	***L. monocytogenes***	***E. coli***
(b) (4)					