FINAL REPORT
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Development of Methodology for Correctly Enumerating Bacteria in High Fat Rendered Animal Co-Products

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Lay Overview of Project and Goals:

Accurate enumeration of bacteria in rendered animal co-products has proven a challenge due to the high fat nature of the materials. Fat coats bacterial cells and prevents proper dispersion in aqueous based bacterial diluents used in serial dilution. If each dilution cannot allow even and equal distribution of the bacteria throughout the diluent, extreme errors in final enumeration occur. Unless accurate methodology can be created to ensure accuracy in bacterial counts, all microbiological studies on rendered animal co-products become suspect. Attempts to complete the thermal death time studies to establish a calculated process are dependent on the ability to accurately enumerate bacteria in rendering samples. Previous studies in this laboratory proved that replicates do not match – that the same sample provides huge variations in bacterial counts among replicates. The results of previous bacterial enumeration trials had extremely high, and unacceptable, standard error. This project is designed as exploratory study to investigate a number of techniques for improving accuracy of bacterial counts in high fat, animal co-products.

Progress since Last Report (since April 1, 2009):

Lard and peanut butter have been used as a test medium for the initial screening studies. After applying a lecithin diluent in the microbial enumeration of inoculated lard proved successful, another high fat food system was assessed. Peanut butter was used because it represents a simple fat and protein matrix to test this concept. Peanut butter was inoculated with *Escherichia coli* and enumerated using a novel technique involving the addition of lecithin to phosphate buffer dilution bottles ADM Ultralec® Deoiled Lecithin, a granular food grade emulsifier, was added to phosphate dilution water in order to promote separation and emulsification of fat globules in the sample. Two commercially available brands of peanut butter were tested in three groups: a control group containing *E.* coli in phosphate buffer, an inoculated peanut butter sample in phosphate buffer, and an inoculated peanut butter sample in the novel granular lecithin diluent. After multiple trials and adjusting experimental factors such as concentration and mixing techniques, data was obtained that indicated increased accuracy in enumerating the bacteria in the high fat food system. Current testing is applying this technique to peanut butter inoculated with *Staphylococcus aureus* and will then be applied to ground beef and animal co-products.

An inoculated peanut butter sample was prepared using a stomacher. The ability of the stomacher to disperse a liquid in a sample of peanut butter was verified by adding 1mL of commercially available food coloring to a 49g sample of peanut butter and testing for visual dispersion of the food coloring using different power and time settings. These tests indicated the settings of high power and eight minutes time were most effective at dispersing the dye. The inoculated sample

was then prepared by aseptically transferring 49g of peanut butter into a sterile WhirlPak Stomacher bag. One milliliter of *E. coli* was dispensed and the bag was sealed. The sample was stomached on high for four 120s cycles, and the bag was turned after each cycle.

The new diluent developed to promote fat globule separation and, theoretically, increase the accuracy of microbial enumeration consists of ADM Ultralec® Deoiled Lecithin, a commercially available food grade emulsifying agent. Ultralec® is a yellow, granular lecithin that was added to phosphate water in the preparation of dilution bottles. These bottles were autoclaved and stored at room temperature. The sample was then tested using three plating/dilution groups: a bacteria control of *E. coli* in phosphate buffer, a second control to demonstrate the effect of no emulsifier (inoculated peanut butter sample in phosphate water), and an experimental group (inoculated peanut butter sample in a lecithin diluent). All three groups were serially diluted to 10^{-9} and plated on duplicate on both Violet Red Bile Agar (to isolate *E. coli*) and Standard Plate Count Agar (to determine the total microbial count of the sample). Results indicated similar counts on the control (E. coli + Phosphate Buffer) and the experimental (PB Sample [w/ E. coli] + Granular Lecithin Buffer) but erratic counts on the standard buffer without the emulsifying agent.

Peanut Butter Brand 1 *

	Rep 1		Rep 2		Rep 3	
E. coli + Phosphate Buffer						
Dilution	VRBA	SPC	VRBA	SPC	VRBA	SPC
10-2	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC
10-3	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC
10-4	35/28	118/122	TNTC/TNTC	TNTC/TNTC	201/191	TNTC/TNTC
10-5	2/3	19/23	281/271	TNTC/TNTC	23/19	198/189
10-6	0/0	0/0	28/33	103/101	3/1	22/19
10-7	0/0	0/0	3/3	10/12	0/0	0/0
10-8	0/0	0/0	0/0	0/0	0/0	0/0
10-9	0/0	0/0	0/0	0/0	0/0	0/0
PB Sample (w/ E. coli) + Phosphate Buffer						
Dilution	VRBA	SPC	VRBA	SPC	VRBA	SPC
10-2	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC
10-3	TNTC/68	TNTC/281	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC
10-4	8/21	31/41	TNTC/138	TNTC/TNTC	256/221	TNTC/198
10-5	4/1	3/1	110/201	281/14	75/108	101/47
10-6	0/0	5/1	7/12	10/18	7/19	TNTC/18
10-7	1/2	3/8	13/TNTC	22/108	0/0	101/23
10-8	0/0	0/0	8/3	10/10	0/0	2/1
10-9	0/0	0/0	8/3	11/17	0/0	0/0
PB Sample (w/ E. coli) + Granular Lecithin Buffer						
Dilution	VRBA	SPC	VRBA	SPC	VRBA	SPC
10-2	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC
10-3	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC
10-4	39/37	127/133	TNTC/TNTC	TNTC/TNTC	212/202	TNTC/TNTC
10-5	4/3	11/14	271/278	TNTC/TNTC	21/22	189/194
10-6	0/0	0/0	27/31	107/104	2/2	19/20
10-7	0/0	0/0	2/3	10/11	0/0	0/0
10-8	0/0	0/0	0/0	0/0	0/0	0/0

10-9	0/0	0/0	0/0	0/0	0/0	0/0

Peanut Butter Brand 2*

	Rep 1		Rep 2		Rep 3	
E. coli + Phosphate Buffer						
Dilution	VRBA	SPC	VRBA	SPC	VRBA	SPC
10-2	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC
10-3	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC
10-4	108/99	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	96/93	198/187
10-5	10/9	112/115	111/114	TNTC/TNTC	8/9	20/19
10-6	0/0	12/14	9/12	184/178	0/0	2/3
10-7	0/0	0/0	0/0	17/19	0/0	0/0
10-8	0/0	0/0	0/0	0/0	0/0	0/0
10-9	0/0	0/0	0/0	0/0	0/0	0/0
PB Sample (w/ E. coli) + Phosphate Buffer						
Dilution	VRBA	SPC	VRBA	SPC	VRBA	SPC
10-2	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC
10-3	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC
10-4	TNTC/TNTC	TNTC/TNTC	207/288	TNTC/200	211/220	198/122
10-5	98/213	TNTC/108	96/TNTC	19/133	18/108	27/39
10-6	TNTC/12	12/15	12/14	2/2	TNTC/22	110/28
10-7	17/3	11/2	0/0	3/9	3/4	3/3
10-8	8/2	0/0	0/0	0/0	0/0	0/0
10-9	0/0	0/0	0/0	0/0	0/0	0/0
PB Sample (w/ E. coli) + Granular Lecithin Buffer						
Dilution	VRBA	SPC	VRBA	SPC	VRBA	SPC
10-2	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC
10-3	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC
10-4	106/101	TNTC/TNTC	TNTC/TNTC	TNTC/TNTC	100/98	193/188
10-5	10/11	117/120	109/113	TNTC/TNTC	10/11	20/19
10-6	0/0	12/10	10/11	180/176	0/0	2/1
10-7	0/0	0/0	0/0	17/15	0/0	0/0
10-8	0/0	0/0	0/0	0/0	0/0	0/0
10-9	0/0	0/0	0/0	0/0	0/0	0/0

*TNTC = too numerous to count which is equivalent to any colony count over 300 per plate.

Project proposals were submitted to The Peanut Foundation and to USDA for funding of continued work on this project. Unfortunately, both grant requests were rejected.

Significance to the rendering industry:

Results indicated that use of the emulsifier allowed more accurate enumeration of bacteria in the presence of high fat. Development of improved enumeration techniques for use in rendered animal co-products will allow more accurate microbial testing. With new regulations upcoming related to microbial validation, the need for accurate microbial enumeration methodology is urgent. The FDA Reportable Food Registry may have huge impact on the rendering industry and it is imperative that models be developed to predict pathogen death rate, D and Z values and other aspects of a calculated thermal process. Earlier work by ACREC researchers on the calculated thermal death time process were halted due to faulty bacterial enumeration methodology with the high fat rendering products. This project will allow researchers to solve those issues and continue their quest to determine calculated thermal processes for the rendering industry.