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A FOOD TESTING LABORATORY SINCE 1967

February 21, 2007

Mr. Carl Knueven
Jones-Hamilton Co.
30354 Tracy Rd.
Walbridge, OH 43465
Tel: 419-666-9838
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Dear Carl,

Please find attached a report for the project entitled "Microbiological Challenge Study of Salad Dressing Product Acidified with Sodium Acid Sulfate and Acetic Acid."

Please let us know if you have any questions. We at ABC Research appreciate this opportunity to work with you and Jones-Hamilton Co.

Best Regards,

James E. (Ken) Kennedy, Ph.D.
Vice President, Research Microbiology
ABC Research Corp.

Enclosure

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RESEARCH PROJECT REPORT RESEARCH
MICROBIOLOGY DEPARTMENT

DATE: February 21, 2007

PREPARED FOR: Jones-Hamilton Co.

CLIENT CONTACT: Mr. Carl Knueven

TITLE: Microbiological Challenge Study of Salad Dressing Product Acidified with Sodium Acid Sulfate and Acetic Acid.

OBJECTIVE: To determine the microbiological stability of a salad dressing product acidified with sodium acid sulfate (SAS) and a combination of SAS and vinegar during ambient temperature storage when challenged with selected potential spoilage microorganisms (i.e., *Lactobacillus spp*. and *Zygosaccharomyces bailii*).

EXPERIMENTAL APPROACH:

A. TEST PRODUCTS

The client provided sufficient quantity of most ingredients for fabricating ca. 4 lbs each of two Ranch salad dressing formulations for the study. Both formulations were acidified to a pH of 3.5; one with only SAS (pHase®) and the other with a combination of SAS and vinegar (i.e., acetic acid). The product ingredients were shipped to ABC Research Corp where the salad dressing formulations were fabricated using the ingredients provided by the client as well as additional ingredients provided by ABC Research Corp.

The formulations of each product variable are attached. The pH of each product formulation was measured immediately after fabrication and the pH adjusted to 3.5 ± 0.02 using 1 N sodium hydroxide as indicated.

B. TEST MICROORGANISMS

The following microorganisms were used in the study: *Lactobacillus fermentum* (ATCC #14931), *Lactobacillus fructivorans* (ATCC #8288), *Lactobacillus brevis* (ATCC #8287), *Zygosaccharomyces bailii* (ATCC #36946), and *Zygosaccharomyces bailii* (ATCC #36947). All of these strains are considered common spoilage microorganisms of salad dressing products.

Each *Lactobacillus* culture was individually propagated via at least two serial transfers (each incubated at 30°C for 48 h) in acidified MRS Broth (supplemented 0.5% fructose) before the experiment. For each culture (ca. 10 ml), bacterial cells were harvested by centrifugation at 10,000 x g for 10 min., washed twice with sterile Butterfield's Phosphate Buffer, pH 7.2 (BPB) and resuspended in 10 ml of BPB. The final suspension of each culture was combined and

resuspended in BPB to obtain a cell suspension containing equal concentrations of each strain. Preliminary culturing trials were performed to determine the culture counts and proportions of each culture required in preparing the final cocktail. A final inoculum cocktail was prepared by diluting the mixed suspension in sterile BPB to obtain ca. 10 ml of a suspension with an approximate cell density of 5 x 10^4 CFU/ml. Preliminary and final inoculum suspensions were enumerated on the appropriate media (see section D).

Each *Zygosaccharomyces* culture was individually propagated via at least two serial transfers (each incubated at 30°C for 72 h) in Sabouraud Dextrose Broth (supplemented with 0.7% glucose, 0.1% fructose, 0.5% salt, and 0.5% acetic acid) before the experiment. For each culture (ca. 10 ml), yeast cells were harvested by centrifugation at 10,000 x g for 10 min., washed twice with sterile BPB and resuspended in 10 ml of BPB. The final suspensions of each culture were combined and resuspended in BPB to obtain a cell suspension containing equal concentrations of each strain. Preliminary culturing trials were performed to determine the culture counts and proportions of each culture required in preparing the final cocktail. A final inoculum cocktail was prepared by diluting the mixed suspension in sterile BPB to obtain ca. 10 ml of a suspension with an approximate cell density of 5 x 10^4 CFU/ml. Preliminary and final inoculum suspensions were enumerated on the appropriate media (see section D).

C. SAMPLE PREPARATION AND INOCULATION

For each product formulation (i.e., acidified with pHase only and acidified with pHase and vinegar), the product container was thoroughly mixed and twenty-five gram portions were aseptically placed into sterile plastic containers (2 oz.) for inoculation and storage. For inoculated sample sets, each sample portion was inoculated in the container by adding 0.1 ml of the designated final cocktail inoculum in 0.01 ml droplets across the surface of the product and thoroughly mixing it into the sample. The inoculation technique resulted in a *Lactobacillus spp*. count of ca. 400 CFU/g of product and a *Zygosaccharomyces* count of 1,200 CFU/g of product.

For each product formulation, an uninoculated set of samples was likewise prepared to monitor any development of indigenous microbial contaminants.

D. SAMPLE STORAGE AND SAMPLE ANALYSES

Two sample containers of each inoculated product formulation were microbiologically analyzed for *Lactobacillus spp*. and *Zygosaccharomyces* after inoculation/packaging (time-0). After inoculation, time-0 samples were analyzed, and the remaining containers were stored at 25°F and analyzed after 1, 2, 3, 4, 6, 8, 10, and 12 weeks of storage. One sample container of each uninoculated product was also analyzed at each designated storage time.

Each sample was thoroughly stirred and the entire contents were aseptically placed in a stomacher bag along with BPB diluent to effect a 1:10 dilution. Samples were stomached for 1 min. and the homogenate serially diluted in BPB as required.

Lactobacillus inoculated samples were analyzed for the inoculum using pour plating procedures with MRS agar (supplemented 1% fructose). MRS plates were incubated anaerobically

(and/or in a CO_2 enriched atmosphere) at 30°C for 5 days. *Zygosaccharomyces* inoculated samples were analyzed for the inoculum using pour plating procedures with PDA agar (supplemented with antibiotics and 1% fructose). PDA plates were incubated aerobically at 30°C for 5 days. Uninoculated samples were analyzed for indigenous lactobacilli (e.g., lactic acid bacteria) and yeasts using the respective methods described previously. All microbial counts were expressed as colony forming units (CFU) per gram.

RESULTS:

The results for *Lactobacillus* and *Zygosaccharomyces* inoculated into both Ranch Dressing product formulations (i.e., acidified with pHase only and acidified with pHase and vinegar) are presented in Table 1. *Lactobacillus* counts in inoculated samples of both product formulations decreased progressively over 3 weeks of storage at 25°C to undetectable levels (i.e., <10 CFU/g). This represented a mean decrease of greater than ca. 1.6 $\log_{10}$ CFU/g. *Lactobacillus* counts remained undetectable through 12 weeks of storage and there was no evidence of a growth trend in either Ranch Dressing formulation (i.e., acidified with pHase only and acidified with pHase and vinegar) during 12 weeks of 25°C storage.

Zygosaccharomyces counts in inoculated samples of both product formulations had decreased to undetectable levels (i.e., <10 CFU/g) after 1 week of storage at 25°C. This represented a mean decrease of greater than ca. 2 $\log_{10}$ CFU/g. *Zygosaccharomyces* counts remained undetectable at most storage intervals through 12 weeks although *Zygosaccharomyces* was recovered at relatively low levels (i.e., ca. one log-unit lower than initial counts) after 2 weeks of storage for the product acidified with pHase alone as well as after 2 and 8 weeks of storage for the product acidified with pHase and vinegar. Although there was some sporadic recovery of *Zygosaccharomyces* during storage for both product formulations, there was no evidence of a growth trend in either Ranch Dressing formulation (i.e., acidified with pHase only and acidified with pHase and vinegar) during 12 weeks of 25°C storage.

Indigenous lactic acid bacteria (LAB) and yeasts counts for both of the uninoculated product formulations are presented in Table 2. LAB and yeasts counts were below detectable levels (i.e., <10 CFU/g) at time-0 and remained undetectable through 12 weeks of storage at 25°F.

PREPARED BY: _____

James E. (Ken) Kennedy, Ph.D.
Vice President, Research Microbiology
ABC Research Corp.

Table 1. Results for Inoculated Ranch Dressing Acidified with pHase and/or Vinegar during Storage at 25°C.

Storage Time (weeks)		**Acidified with pHase alone**				**Acidified with pHase and Vinegar**			
		Lactobacillus		***Zygosaccharomyces***		***Lactobacillus***		***Zygosaccharomyces***	
		CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g	CFU/g	Log_{10} CFU/g
0	rep. 1	500	2.70	1,000	3.00	540	2.73	1,500	3.18
	rep. 2	340	2.53	1,100	3.04	300	2.48	1,100	3.04
	Mean		**2.62**		**3.02**		**2.60**		**3.11**
1	rep. 1	120	2.08	<10	<1.00	160	2.20	<10	<1.00
	rep. 2	100	2.00	<10	<1.00	230	2.36	<10	<1.00
	Mean		**2.04**		**<1.00**		**2.28**		**<1.00**
	Change		**-0.58**		**<-2.02**		**-0.32**		**<-2.11**
2	rep. 1	140	2.15	100	2.00	320	2.51	<10	<1.00
	rep. 2	170	2.23	120	2.08	320	2.51	<10	<1.00
	Mean		**2.19**		**2.04**		**2.51**		**<1.00**
	Change		**-0.43**		**-0.98**		**-0.10**		**<-2.11**
3	rep. 1	<10	<1.00	10	1.00	<10	<1.00	<10	<1.00
	rep. 2	<10	<1.00	<10	<1.00	<10	<1.00	<10	<1.00
	Mean		**<1.00**		**1.00**		**<1.00**		**<1.00**
	Change		**<-1.62**		**-2.02**		**<-1.60**		**<-2.11**
4	rep. 1	<10	<1.00	<10	<1.00	<10	<10	<10	<1.00
	rep. 2	<10	<1.00	<10	<1.00	<10	<10	<10	<1.00
	Mean		**<1.00**		**<1.00**		**<1.00**		**<1.00**
	Change		**<-1.62**		**<-2.02**		**<-1.60**		**<-2.11**
6	rep. 1	<10	<1.00	<10	<1.00	<10	<1.00	<10	<1.00
	rep. 2	<10	<1.00	<10	<1.00	<10	<1.00	<10	<1.00
	Mean		**<1.00**		**<1.00**		**<1.00**		**<1.00**
	Change		**<-1.62**		**<-2.02**		**<-1.60**		**<-2.11**
8	rep. 1	<10	<1.00	<10	<1.00	<10	<10	250	2.40
	rep. 2	<10	<1.00	<10	<1.00	<10	<10	310	2.49
	Mean		**<1.00**		**<1.00**		**<1.00**		**2.44**
	Change		**<-1.62**		**<-2.02**		**<-1.60**		**-0.66**
10	rep. 1	<10	<1.00	<10	<1.00	<10	<10	10	1.00
	rep. 2	<10	<1.00	<10	<1.00	<10	<10	<10	<1.00
	Mean		**<1.00**		**<1.00**		**<1.00**		**1.00**
	Change		**<-1.62**		**<-2.02**		**<-1.60**		**-2.11**
12	rep. 1	<10	<10	<10	<10	<10	<10	<10	<10
	rep. 2	<10	<10	<10	<10	<10	<10	<10	<10
	Mean		**<1.00**		**<1.00**		**<1.00**		**<1.00**
	Change		**<-1.62**		**<-2.02**		**<-1.60**		**<-2.11**

Notes: 1) Change = (mean log CFU/g at subject time,) - (mean log CFU/g at time-0).
2) Study was initiated on 11/3/06.

Table 2. Results for Uninoculated Ranch Dressing Acidified with pHase and/or Vinegar during Storage at 25°C.

Storage Time (weeks)	Acidified with pHase alone		Acidified with pHase and Vinegar	
	Lactic Acid Bacteria	Yeast	Lactic Acid Bacteria	Yeast
	CFU/g	CFU/g	CFU/g	CFU/g
0	<10	<10	<10	<10
1	<10	<10	<10	<10
2	<10	<10	<10	<10
3	<10	<10	<10	<10
4	<10	<10	<10	<10
5	<10	<10	<10	<10
6	<10	<10	<10	<10
8	<10	<10	<10	<10
10	<10	<10	<10	<10
12	n/d	n/d	<10	<10

Notes: 1) Study was initiated on 11/3/06.
2) n/d = no data due to a lab error.

RANCH DRESSING (With pHase Only) - pH 3.50

	Ingredient	%	Grams per batch
	Water	41.850	627.720
	pHase	**0.600**	**9.000**
	Soybean oil	41.542	623.130
	buttermilk powder	4.000	60.000
	Xanthan Gum	0.080	1.200
	Starch	1.680	25.200
	Sorbic Acid	0.200	3.000
	EDTA	0.007	0.105
	Salt	0.500	7.500
	Sugar	1.500	22.500
	Seasoning and other spices	5.260	78.900
	Egg and Egg yolk	2.700	40.500
	Vinegar (120 grain)	**0.000**	**0.000**
	Paprika spice concentrate	0.080	1.200
	Oleoresin black pepper	0.003	0.045
	Total	**100.002**	**1500.000**

Processing Method:

1. Combine and mix the dry ingredients (pHase, buttermilk powder, sugar, salt, EDTA, sorbic acid, starch and gum) in a Hobart mixer
2. Mix in the egg and water
3. Add oil slowly while mixing to form a coarse emulsion
4. Add spices and vinegar
5. Add the acidulant (pHase)
6. Adjust pH to 3.5 (and/or the same as the other dressing variable) if indicated
7. Fill into appropriate container

RANCH DRESSING (With pHase and Vinegar) - pH 3.50

	Ingredient	%	Grams per batch
	Water	41.15	617.220
	pHase	**0.50**	**7.500**
	Soybean oil	41.542	623.130
	buttermilk powder	4.00	60.000
	Xanthan Gum	0.08	1.200
	Starch	1.68	25.200
	Sorbic Acid	0.20	3.000
	EDTA	0.007	0.105
	Salt	0.50	7.500
	Sugar	1.50	22.500
	Seasoning and other spices	5.26	78.900
	Egg and Egg yolk	2.70	40.500
	Vinegar (120 grain)	**0.80**	**12.000**
	Paprika spice concentrate	0.08	1.200
	Oleoresin black pepper	0.003	0.045
	Total	**100.002**	**1500.000**

Processing Method:

1. Combine and mix the dry ingredients (pHase, buttermilk powder, sugar, salt, EDTA, sorbic acid, starch and gum) in a Hobart mixer
2. Mix in the egg and water
3. Add oil slowly while mixing to form a coarse emulsion
4. Add spices and vinegar
5. Add the acidulant (pHase)
6. Adjust pH to 3.5 (and/or the same as the other dressing variable) if indicated
7. Fill into appropriate container