ANNEX XI-A:

IN VITRO HUMAN FAECAL MICROFLORA FERMENTATION STUDIES PERFORMED ON PHOSPHATED DISTARCH PHOSPHATE PRODUCED FROM EITHER WHEAT OR POTATO STARCH

WORK UNDERTAKEN FOR MGP INGREDIENTS BY PROFESSOR LILLIAN THOMPSON, UNIVERSITY OF TORONTO.

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Dear Dr. Maningat:

Per your request, I am re-issuing the attached report on "In Vitro Digestion and Fermentation of Resistant Starch: Final Report"

Sincerely yours,

Lilian U. Thompson

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IN VITRO DIGESTION AND FERMENTATION OF RESISTANT STARCH:

FINAL REPORT

Lilian U. Thompson, Ph.D.
Professor

1. Objective

To determine the rate of fermentation of two resistant starches (potato and wheat) using in vitro method with human fecal inoculum.

2. Methods

2.1 In vitro digestion

Starch digestion under small intestinal conditions was conducted in vitro according to our standard methods (1-2).

Briefly, duplicate 20 g resistant wheat starch (Fibersym 70 Lot No. 7843) and resistant potato starch (Fibersym 80 ST Lot No. 04-420) were dispersed in 80 ml distilled water and placed in boiling water bath for 30 min. After cooling, the dispersions were adjusted to pH 2.0, pepsin-HCl solution was added and then incubated for 3 hours in a water bath at 37°C. Aliquots of the pepsin digests were placed in duplicate dialysis bag containing pancreatin-bile solution plus bile extract in 0.1 M $NaHCO_3$, and merthiolate solution. They were then digested and dialyzed against sodium bicarbonate solution (pH7.5) for 12 hours at 37°C, with changes in dialysate every 3 hours. A blank control was tested at the same time as the starches.

To determine the amount of 0.5 M $NaHCO_3$ needed in the dialysate, a 20 g aliquot of each pepsin digest, to which pepsin-pancreatin bile mixture had been added, was titrated to pH 7.5 with the $NaHCO_3$ solution. This calculated volume of $NaHCO_3$ was then made up to 100 mL with deionized water and used for dialysis.

After digestion, the dialysis retentates were freeze dried and weighed. The duplicate freeze dried retentates were pooled and aliquots were used for in vitro fermentation.

Weights were corrected for the weight obtained with the blank control.

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2.2 In vitro fermentation

This was conducted using methods established in our laboratory and previously described (3-11).

Briefly, freeze dried starch dialysis retentates were weighed into 100 mL serum bottles and the fermentation medium (40 mL) was added. Blank control samples contain only the medium. The serum bottle contents were reduced using the conditions and reducing agents described by Goering and van Soest (12), sealed with a butyl rubber stopper crimped with a metal seal, and stored overnight at 4°C. One to two hours prior to inoculation with fecal inoculum, the bottles were put into a water bath at 37°C.

Feces was collected from a healthy individual into a tared blender which was continuously flashed with CO_2, diluted with deoxygenated distilled water (66.6 g wet feces/liter), blended for 30 sec, squeezed through 41-um Nitex membrane, and filtered through glass wool to remove fibrous particles. Ten mL of this inoculum was then injected through the septum of the serum bottle. Fecal contents and containers were kept under a continuous flow of CO_2 at all times. Serum bottles were incubated in a water bath at 37°C with swirling at regular intervals. Duplicate samples were removed for analysis at 0, 4, 8, 12 and 24 hours.

Immediately after removal of the serum bottles from the water bath, gas production was measured. A 60-mL syringe with a three-way stopcock and needle was used to sample gas contents in the serum bottle to determine total gas production as previously described (1). The serum bottle was then opened and 1 mL of 1% copper sulfate solution was added to inactivate the microorganisms. An aliquot was taken from each flask, centrifuged, filtered through 0.22 Millipore filter and analyzed for short chain fatty acids (SCFA) using HPLC method with diode array detector.

The SCFA values were corrected for the values obtained with the blank control.

3. Results and Discussion

Table 1 shows the starch residue recovery after in vitro starch digestion. Under the conditions of the experiment, about 74% and 64% of the wheat starch and potato starch, respectively, were resistant to digestion. The freeze dried residues were not analyzed for moisture content and the resistant starch may be overestimated. However, freeze drying of starchy foods in our previous studies usually result in about 1% moisture content, which may approach the concentration in the undigested starch.

Total gas production increased almost linearly over time, indicating that fermentation occurred during the 24 hour period (Table 2). The two starches had similar gas production rates.

The SCFA production per g resistant starch fermented also increased over time (Table 3). Wheat starch produced relatively higher amounts of SCFA than potato starch, indicating faster rate of fermentation or higher fermentability, but the difference may not be statistically significant. The main SCFA were acetic acid (C2), propionic acid (C3) and butyric acid (C4), which continuously increased during the entire 24 hours but tended to level off after 12 hours. The fractional molar

ratios of C2, C3 and C4 were 0.516/0.186/0.228 for wheat starch and 0.577/0.200/0.233 for potato starch at the end of fermentation. Hence the starches produced C2 as the major SCFA and relatively higher amounts of C4 than C3. This was in contrast to the fermentation results of several purified dietary fiber sources (pectin, tragacanth, psyllum, guar, soy fiber, cellulose), which generally produced more C3 than C4 (4). Formic acid (C1), isobutyric acid (iC4), valeric acid (C5) and isovaleric acid (iC5) were initially produced during fermentation but underwent deterioration by 24 hours, in agreement with our previous observations on raw starch fermentation (5). Much of the starch were fermented to SCFA within 12 hours.

The total SCFA production of the two resistant starches was less than the previously reported SCFA production after fermentation of several raw starches (5) and dietary fiber (4). This indicates that the resistant starch samples are less fermentable than the other raw starches or fiber. However, it could also be due to differences in the activity of colonic bacteria used in the fermentation.

4. Conclusion

The wheat and potato starch samples had high concentrations of resistant starch, which can be fermented by colonic bacteria to SCFA and gas. Wheat starch tended to have higher amount of resistant starch than potato starch and produced similar amount of gas but more SCFA than potato starch after 24 hours fermentation. C2 was the major SCFA that was produced but slightly higher amounts of C4 was produced than C3.

5. References

1. Trinidad T, Wolever TMS, Thompson LU.1996. Availability of calcium for absorption in the small intestine and colon from diets containing available and unavailable carbohydrates: an in vitro assessment. Int J Food Sci Nutr. 47:83-88

2. Trinidad T, Wolever TM, Thompson LU 1996. Availability of calcium from dairy foods as affected by dietary fibers. Trace Elem. Electr. J.13: 123-125

3. McBurney MI, Thompson LU. 1987. Effect of human fecal inoculum on in vitro fermentation variables. Br J Nutr 58:233-243

4. McBurney MI, Thompson LU, 1989. In vitro fermentabilities of purified fiber supplements. J Food Sci 54: 347-350

5. McBurney MI, Cuff DJ, Thompson LU. 1990. Rates of fermentation and short chain fatty acid and gas production of six starches by human fecal microbiota. J Sci Food Agric 50: 79-88

6. McBurney MI, Thompson LU. 1989. Effect of human faecal donor on in vitro fermentation variables. Scand J Gastroenterol 24:359-367

7. McBurney MI, Thompson LU. 1988. Dietary fiber and energy balance: integration of the human ileostomy and in vitro fermentation models. Anim Feed Sci Technol 23:261-275

8. McBurney MI, Thompson LU, Cuff DJ, Jenkins DJ. 1988. Comparison of ileal effluents, dietary fibers, and whole foods in predicting the physiological importance of colonic fermentation. Am J Gastroent. 83:536-540

9. McBurney MI, Thompson LU. 1987. Colonic fermentation of some breads and its implication for energy availability in man. Nutr Res 7:1229-1241

10. McBurney MI, Thompson LU. 1990.Fermentative characteristics of cereal brans and vegetable fibers. Nutr Cancer. 13(4):271-80.

11. McBurney MI, Thompson LU.1991. Dietary fiber and total enteral nutrition: fermentative assessment of five fiber supplements. J Parenter Enteral Nutr. 15(3):267-70.

12. Goering HK, Van Soest PJ. 1970. Forage Fiber Analyses (Apparatus, Reagents, Procedures and Some Applications). USDA, Washington DC

Table 1. Percent resistant starch (based on recovered residues) after pepsin-pancreatin digestion of wheat and potato starches.

Sample	% Resistant Starch
Wheat starch	73.7 ± 1.0
Potato starch	63.8 ± 0.6

Mean ± SEM; n=4

Table 2. Cumulative total gas production after fermentation of resistant starch at various times (mmol/g starch)

	Fermentation Time (hours)			
Sample	4	8	12	24
Wheat starch	78.0 ± 0	118.5 ± 5.8	153.3 ± 5.8	214.2 ± 2.8
Potato starch	77.9 ± 0	112.8 ± 11.6	150.4 ± 8.7	214.2 ± 14.5

Mean ± SEM; n=2

Table 3. Cumulative short chain fatty acid (SCFA) production after fermentation of resistant starch at various times (mmol/g starch)

		Fermentation Times (hours)			
	SCFA	4	8	12	24
Wheat starch *	C1	0.50 ± 0.08	0.69 ± 0.10	0.76 ± 0.15	0.00 ± 0.00
	C2	1.85 ± 0.35	2.87 ± 0.27	3.44 ± 0.03	3.85 ± 0.21
	C3	0.39 ± 0.02	1.05 ± 0.03	1.10 ± 0.04	1.22 ± 0.04
	iC4	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	C4	0.40 ± 0.10	0.93 ± 0.08	1.32 ± 0.00	1.50 ± 0.05
	iC5	0.04 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	C5	0.05 ± 0.03	0.04 ± 0.00	0.04 ± 0.01	0.01 ± 0.01
	Total	3.25 ± 0.60	5.58 ± 0.48	6.66 ± 0.21	6.58 ± 0.30
Potato starch **	C1	0.65 ± 0.02	0.63 ± 0.07	0.35 ± 0.02	0.00 ± 0.00
	C2	2.12 ± 0.04	2.87 ± 0.17	2.93 ± 0.18	3.52 ± 0.07
	C3	0.41 ± 0.01	0.93 ± 0.04	1.21 ± 0.06	1.22 ± 0.06
	iC4	0.02 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	C4	0.47 ± 0.00	0.83 ± 0.07	1.06 ± 0.01	1.36 ± 0.01
	iC5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	C5	0.08 ± 0.00	0.03 ±0.00	0.02 ± 0.00	0.00 ± 0.00
	Total	3.75 ± 0.07	5.28 ± 0.35	5.58 ± 0.21	6.10 ± 0.14

Mean ± SEM; n= 2

SCFA= short chain fatty acids; C1= formic acid; C2= acetic acid; C3= propionic acid; iC4-isobutyric acid; C4= butyric acid; iC5= isovaleric acid; C5= valeric acid

Fractional molar ratio of C2/ C3/ C4 at 24 hours * 0.586/ 0.186/ 0.228

** 0.577/ 0.200/ 0.223