Research Article

Starch-entrapped microspheres extend *in vitro* fecal fermentation, increase butyrate production, and influence microbiota pattern

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Previous research has revealed that waxy corn starch which has been entrapped in a matrix of electrostatically cross-linked alginate, shows a slow digestion rate such that much of the starch may reach the colon; thus making this a new type of resistant starch. The purpose of this research was to test the fermentative properties of starch-entrapped microspheres using a batch fecal fermentation method. Fermentation of starch-entrapped microspheres showed significantly lower rates of gas production compared to waxy corn starch, and showed significant increases in total SCFAs during the latter stages of fermentation (24 – 48 h), whereas waxy corn starch did not. Cooking the starch-entrapped microspheres increased the amount of SCFAs and the molar fraction of butyrate produced during fermentation. Bacterial fingerprinting revealed that uncooked starch-entrapped microspheres have a unique effect on the microbiota that is different from waxy corn starch alone, but cooking causes a shift toward a pattern more closely resembling that of the starch. Starch-entrapped microspheres may deliver slowly fermentable carbohydrate to the colon, with the ability to influence the microbiota. Further human studies are required to determine whether these characteristics occur *in vivo*.

Keywords: Butyrate / Colon / Encapsulation / Resistant starch / Short chain fatty acids

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1 Introduction

When dietary fiber reaches the colon, it can be fermented by colonic bacteria, producing, among other products, SCFAs (mainly acetate, propionate, and butyrate). SCFAs are beneficial to colonic health because they inhibit the growth of pathogenic bacteria [1 – 3] decrease the production of secondary bile acids [4], and increase mineral absorption [5, 6]. Acetate and propionate can be absorbed into the blood stream and influence the release of insulin from the liver [7], and butyrate provides an important source of energy for colonic epithelial cells. Butyrate is also important in cell differentiation and proliferation [7], and helps modulate inflammation due to its influence on proinflammatory cytokines [3, 8].

Unfortunately with regards to colonic health, as carbohydrates are metabolized, bacterial fermentation results in lower levels of SCFAs and an increase in undesirable compounds such as phenols, branched chain fatty acids, and ammonia from protein fermentation [9]. Compared to other regions, the distal colon is the most prone to disease, presumably because of the lack of fermentable carbohydrate and increased production of toxic metabolites from putrefactive fermentation [10]. Therefore, intake of slowly fermentable fibers may help increase beneficial fermentative products in the distal regions of the colon and improve colonic health.

Alginate, a polysaccharide of guluronic and mannuronic acids, forms stable gels in the presence of a divalent cation. Calcium alginate microspheres have been successfully used to entrap numerous substances, such as: cells [11], hormones [12], proteins [13], enzymes [14, 15], and drugs [16]. In our laboratory, we have developed a method of

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Abbreviations: LH-PCR, length heterogeneity of PCR; **OTU,** operational taxonomic unit; **PCA,** principle component analysis

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entrapping starch in an alginate matrix. Alginate entrapment of starch results in substantial decreases in both postprandial glucose and insulin concentrations compared to a glucose standard during the first 2 h after consumption. During the third hour after ingestion, starch-entrapped microspheres resulted in significantly higher blood glucose compared to ingestion of a glucose standard, indicating an extended release of glucose [17]. During development of these microspheres, we observed that a considerable amount of starch remained in the microspheres after *in vitro* digestion using the Englyst method [18] and this amount could be manipulated by altering the composition of the microspheres. Thus, it was hypothesized that much of the starch in the microspheres may escape digestion in the small intestine, providing a source of dietary fiber. Unlike most soluble, fermentable fibers and resistant starch, which generally ferment rapidly [19], starch-entrapped microspheres may exhibit the additional benefit of fermenting slowly due to the physical alginate barrier; thus reducing the production of undesirable fermentative products in the distal colon by increasing fermentable carbohydrate in this region. Furthermore, the rate of fermentation may be directly related to the severity of bloating and abdominal distention that is commonly caused by fiber due to rapid gas production. A slow fermentation rate could minimize this undesirable side effect of fiber. The purpose of this study was to use a batch fecal fermentation method to estimate the fermentative properties of starch-entrapped microspheres in the colon.

2 Materials and methods

2.1 Preparation of starch-entrapped microspheres

Suspensions of sodium alginate, waxy corn starch, and water were prepared using amounts shown on the left side of Table 1; the alginate was dissolved first, and then the starch was added. Due to the viscosity of the alginate solution at higher concentrations, small air bubbles tended to become trapped in the solution during stirring; therefore, the solution was placed in a desiccator, and a vacuum was pulled to remove air bubbles. Waxy corn starch was used to minimize retrogradation. With continuous stirring, the suspension was pumped through a 22 gauge hypodermic needle using a peristaltic pump (Tris, Teledyne Isco, Lincoln, NE) into a bath of calcium chloride (4% w/v). The microspheres were kept in the calcium chloride bath for 1–4 h, and then harvested by filtration. After extensive washing, the microspheres were dried in an oven at 40°C for 24 h. This low drying temperature was used to ensure starch was not gelatinized. Blank microspheres were also created using a solution of 2% w/w alginate and no starch.

2.2 *In vitro* upper gastrointestinal digestion

To digest the samples, sufficient starch-entrapped microspheres were weighed into a test tube such that 100 mg equivalent starch would remain after digestion; waxy corn starch (100 mg equivalent starch) was also weighed into a test tube as a control. Two milliliters of water was added to all tubes, and "cooked" samples were heated in boiling water for 20 min, while "uncooked" samples were used as is. Both uncooked and cooked samples were then subjected to *in vitro* digestion [20]. Briefly, samples were suspended in phosphate buffer (20 mM, pH 6.9, containing 10 mM NaCl) and then incubated with 100 μL salivary amylase (40 U/mL in 1 mM calcium chloride; A-1031, Sigma Chemicals, St Louis, MO) for 15 min at 37°C. The pH was then adjusted to 1.5 by the addition of HCl, and 100 μL pepsin (40 U/mL in 20 mM HCl; P-7125, Sigma Chemicals) was added and incubated for 30 min. The pH was adjusted to 6.9 with NaOH, and the samples were incubated at 37°C with 100 μL pancreatin (10 mg/mL in phosphate buffer from above; P-7425, Sigma Chemicals) for 90 min. Following digestion, the microspheres were collected by filtration and thoroughly rinsed with water; waxy corn starch was exhaustively dialyzed against acetic acid (0.1 M) at 4°C. The low temperature and acidic pH were used to prevent further amylolytic activity after the digestion process. The filtrate or dialysate was collected in a flask, and the pH was adjusted to 4.5 with sodium acetate, followed by the addition of amyloglucosidase (20 U). The flasks were incubated at 50°C for 1 h, and then glucose was measured using a col-

Table 1. Formulation of suspension prior to starch entrapment and composition of final starch-entrapped microspheres after drying ($n = 2$)

Suspension			Final composition		
Sodium alginate	Starch	Water	Calcium alginate[a)]	Starch	Moisture
1[b)]	9	90	10.8	83.1 ± 1.8[c)]	6.14 ± 0.20
2	9	89	19.4	75.9 ± 0.7	4.66 ± 0.04
3	9	88	30.0	67.0 ± 2.9	3.02 ± 0.16

a) Calculated by difference, *i.e.*, 100% – (% starch + % moisture).
b) All values expressed as percent, on a wet weight basis.
c) Expressed as percent ± SD, on a wet weight basis.

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orimetric method (GOPOD Format, Megazyme, Wicklow, Ireland).

2.3 *In vitro* lower gastrointestinal fermentation

This study utilized a batch fecal fermentation method to estimate the effects of starch entrapment in the colon. Although this is a model of what actually occurs in the large bowel [21], it provides useful data from which to form hypotheses for *in vivo* studies [22].

Freshly voided feces were collected from three healthy volunteers who had not taken antibiotics in the past 6 months. Because each individual harbors a unique microbial community consisting of complex interactions between bacteria, pooling does pose a risk of creating interactions between bacteria that would not occur under physiological conditions. Nevertheless, pooling has been recommended [23] and utilized by a number of researchers (*e.g.*, [19, 24–26]) to create a general microbiota that is free of anomalous results that may arise from the microbiota from a single individual [27]. Within 2 h of collection, during which time they were kept on ice, feces were pooled and homogenized with anaerobic media (1:15 w/v) [28], and filtered through four layers of cheesecloth under continuous carbon dioxide flushing. The composition of the media was (g/L): potassium phosphate dibasic, 0.282; potassium phosphate monobasic, 0.282; ammonium sulfate, 0.282; sodium chloride, 0.564; magnesium sulfate heptahydrate, 0.115; calcium chloride dihydrate, 0.075; trypticase peptone, 1; yeast extract, 1; sodium carbonate, 4; cysteine hydrochloride, 0.6; and resazurin, 0.0001; the pH was adjusted to 6.2 [28]. Carbon dioxide flushing has been shown to adequately maintain anaerobiosis in *in vitro* fermentation studies (*e.g.*, [29, 30]). The presence of reduced (colorless) resazurin also serves as a visual check of anaerobic conditions. A portion of filtrate was immediately frozen at −40°C for microbial fingerprinting (inoculum) and SCFA determination (zero time reading).

Digested starch-entrapped microspheres from above were transferred to serum tubes; undigested waxy corn starch (100 mg equivalent starch) was weighed into a serum vial as a positive control, and microspheres containing no starch (alginate blank) were used as a negative control. Even though waxy corn starch would not normally represent a substantial source of carbohydrate for bacteria in the large bowel, it was used in this study as a control for three reasons: (i) to observe the effect of entrapment, *i.e.*, the starch used as a control was the same starch as was entrapped; (ii) it represents a rapid and completely fermentable carbohydrate to compare to the starch-entrapped microspheres; (iii) it has been used previously as a control for *in vitro* fermentation studies [30]. For each replicate, four tubes were prepared; 1 for each time period measured (4, 8, 24, and 48 h). Two milliliters of anaerobic media were added to all tubes. Samples to be cooked were submerged in boiling water for 20 min, and then 8 mL of fecal homogenate and 0.2 mL of Oxyrase (Oxyrase for Broth, Oxyrase, Mansfield, OH) were added to all tubes. Oxyrase is an enzyme preparation that consumes oxygen; it was added as an additional measure to ensure anaerobiosis. The vials were sealed anaerobically (with carbon dioxide), and incubated with gentle shaking at 37°C. At specified time intervals, total gas volume was measured using a graduated syringe, and then the tube was opened. One and 0.8 mL of digesta were stored at −40°C for microbial and SCFA analysis, respectively, and the pH was measured.

2.4 Total starch determination

To determine total starch, microspheres were ground in a ball mill (Brinkman, Westbury, NY) at 70% power for 2 min to destroy the alginate network and allow enzymatic degradation and quantification of the starch according to the Total Starch method (Megazyme). The final composition of the dried starch-entrapped microspheres is shown on the right side of Table 1.

2.5 Quantification of short chain fatty acids

For SCFA quantification, 0.2 mL of 25% *meta*-phosphoric acid was mixed with 0.8 mL of fecal slurry. After resting 30 min and centrifuging for 10 min (15 000 *g*), 1 μL of supernatant was injected onto an HP 5890 GC, and SCFAs were separated on a Carbowax C packed column (Supelco no. 11825-U) with an oven temperature of 155°C, injector temperature of 200°C, detector temperature of 200°C, and nitrogen as the carrier gas [31]. SCFAs (acetate, propionate, and butyrate) were quantified by comparing the FID response to external standards.

2.6 Bacterial fingerprinting by length heterogeneity-PCR

For bacterial fingerprinting, duplicate frozen samples were defrosted and total genomic DNA was extracted from samples using Bio101 kit (Qbiogene, Montreal, Quebec) as per the manufacturer's instructions. Extracted DNA (10 ng) was amplified in quadruplicate by PCR by using a fluorescently labeled forward primer 27F (5′-[6FAM] AGAGTTTGATCCTGGCTCA G-3′) and unlabeled reverse primer 338Rk (5′-GCTGCCTCCCGTAGGAGT-3′). Both primers were selected because they are universal primers for bacteria [32]. The reactions were performed using 50 μL (final volume) mixtures containing 1 × PCR buffer, 0.6% BSA, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, 0.2 μM of each primer, and 2 U of Taq DNA polymerase (AmpliTaq Gold, Applied BioSystems, Foster City, CA). The initial denaturation step was 94°C for 3 min, followed by 25 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 2 min, fol-

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lowed by a final extension step that consisted of 72°C for 7 min, and then stored at −20°C in the dark until used (usually less than 1 wk).

The PCR products were separated on an SCE9610 capillary fluorescent sequencer (Spectrumedix LLC, State College, PA) and analyzed with GenoSpectrum™ software (Version 2.01). The software package deconvolves the fluorescence data into electropherograms where the peaks of the electropherograms represent the length heterogeneity of PCR amplicons (LH-PCR) in bp and identify different species, or operational taxonomic units (OTUs), of microbiota. All LH-PCR fingerprinting data were then analyzed using a custom PERL script (Interleave 1.0, BioSpherex LLC) that combines data from several runs, interleaves the various profiles, normalizes the data, calculates the averages for each amplicon size, and determines diversity indices. The normalized peak areas were calculated by dividing an individual peak area by the total peak area in that profile. LH-PCR fingerprint patterns (*i.e.*, presence or absence of certain amplicon peaks) were expressed as stacked histograms of the LH-PCR amplicon normalized abundances and analyzed by visual inspection and principle component analysis (PCA).

2.7 Data analysis

Gas production and SCFA data were analyzed with SAS software (version 9.1, SAS Institute, Cary, NC) using a mixed model analysis of variance. Tukey's multiple comparison adjustment was used for the differences between least-squares means. Statistical significance was defined as $p < 0.05$. PCA using a Bray Curtis similarity metric was used to analyze LH-PCR patterns using MVSP software (Kovach, Wales, UK).

3 Results and discussion

3.1 Composition of starch-entrapped microspheres

After harvesting and drying, the starch-entrapped microspheres resembled white, round spheres that were stable to cooking and autoclaving. The size could be manipulated by changing the needle gauge or using an atomizer to dispense the alginate-starch suspension into the calcium bath; however, only one size (*ca*. 0.8 mm) was used in this study.

The composition of the starch-entrapped microspheres is shown in Table 1. Increasing the alginate content, from 1 to 3% in the suspension used to make the microspheres, decreased the percent of starch in the dried microspheres from 83.1 to 67.0%. This decrease was attributed to a higher amount of alginate in the microspheres; thus providing a more dense matrix surrounding the starch, and potentially leading to a reduced rate of digestion and fermentation. Hereafter, the three types of microspheres described in Table 1 will be referred to by their final percent calcium alginate values.

3.2 *In vitro* upper gastrointestinal digestion

We have previously reported small intestine digestion data for starch-entrapped microspheres using the Englyst method [17]. Because alginate gels are anionic in nature, they are prone to destabilization as pH increases. Therefore, there was some concern that the low pH of 4.5 used in the Englyst assay may have a stabilizing effect on the starch-entrapped microspheres that would not be present under physiological conditions. Therefore, the *in vitro* digestion procedure used in this study was employed because it utilizes a higher pH of 6.9 to approximate *in vivo* conditions, and was designed specifically for digestion of samples for *in vitro* fermentation studies [20].

Starch-entrapped microspheres maintained their integrity despite the higher pH used in this digestion assay, as judged by visual observation and recovery of microspheres by filtration. After *in vitro* digestion, only 4.3, 5.8, and 5.0% of the starch had been digested in the uncooked, and 21.8, 12.4, and 9.4% of the starch had been digested in the cooked 10.8, 19.4, and 30.0% alginate microspheres, respectively, relative to cooked, waxy corn starch (100% digestible). In the cooked microspheres, the amount of starch digested decreased with increasing alginate percentage, as expected; however, this trend was not apparent in the raw microspheres. This may be because (i) raw microspheres represent a theoretical minimum of starch digestion or (ii) the digested starch in the raw samples represents a small amount of starch available to enzymic degradation at the surface of the microsphere.

3.3 *In vitro* lower gastrointestinal fermentation

3.3.1 Initial observations

As with *in vitro* digestion in the upper digestive tract, starch-entrapped microspheres maintained their structural integrity throughout *in vitro* fermentation, and could be harvested by filtration after 48 h of fermentation. The microspheres thus obtained were clear, as opposed to predigested microspheres which were white (due to the starch); indicating that the bacteria were able to utilize the starch, but unable to break down the alginate matrix. This was also observed when alginate microspheres containing no starch were incubated with fecal inocula. In this case, the fermentation profiles (*i.e.*, production of gas and SCFAs) were similar to a blank containing only the inoculum (data not shown). Thus, fecal microbial hydrolases were unable to break down the alginate network. The pore size of alginate microspheres has been reported to be between 5 and 200 nm [33]. Because bacteria are measured in the micrometer range, it is unlikely that they are able to enter the microspheres. This supports a mechanism of digestion and

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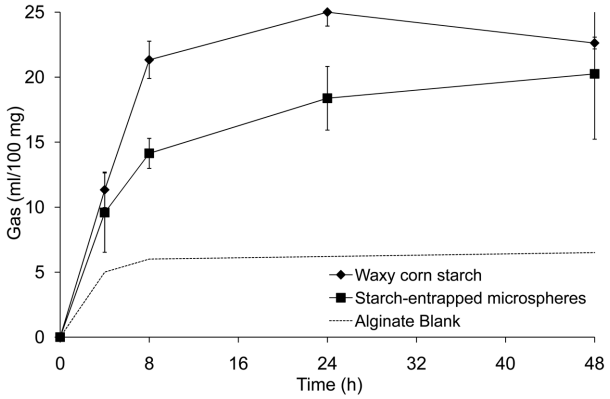

Figure 1. Gas produced during *in vitro* fermentation of the average of all starch-entrapped microspheres and all waxy corn starch samples. Dashed line with no markers indicates alginate blank (expressed as mL/tube). Error bars show SD; some error bars are too small to see; $n = 4$.

colonic bacterial fermentation wherein enzymes diffuse through the alginate matrix of the microsphere, hydrolyze the entrapped carbohydrate, and then the degradation products diffuse out of the matrix to be utilized.

3.3.2 Gas production

The production of gas is an important factor when considering a dietary fiber source. Indeed, much of the aversion to dietary fiber arises from the uncomfortable bloating that can result from high intakes due to rapid distention of the bowel. A dietary fiber that has a low, steady rate of gas production would most likely not result in bloating, because the gases would have time to be slowly expelled through the lungs and anus. Furthermore, the rate of expansion of the gut would be slow rather than rapid which would allow gut to accommodate this change resulting in less discomfort and bloating. Indeed, experimental studies using balloon distention in the rectum and other parts of gastrointestinal tract demonstrate that the rate of balloon distention influence the severity of discomfort.

Neither cooking ($p > 0.05$) nor differences in percent alginate ($p > 0.05$) of the starch-entrapped microspheres significantly affected the production of gas by the microbiota during *in vitro* fermentation; however, entrapment of starch did significantly reduce the rate of microbial gas production when compared to waxy corn starch (Fig. 1). After 8 h of fermentation, microbiota exposed to waxy corn starch produced an average of 21.3 mL of gas, whereas microbiota exposed to starch-entrapped microspheres took 48 h to reach similar values, although there was no difference in the first 4 h. Thus starch-entrapped microspheres may be a source of fermentable substrate without uncomfortable bloating.

3.3.3 pH changes

Figure 2 shows pH changes during *in vitro* fermentation. In microbiota fed cooked waxy corn starch (Fig. 2a), a significantly sharper drop in pH during the first 4 h of fermentation was experienced, compared to bacteria receiving starch-entrapped microspheres ($p < 0.0001$). A significant decrease in pH was observed for all bacteria exposed to starch-entrapped microspheres between 4 and 8 h of fermentation ($p < 0.0001$), but not for those receiving waxy corn starch ($p > 0.05$). After 48 h of fermentation, the pH of all starch-entrapped microsphere samples were statistically indistinguishable ($p > 0.05$). The observed pH drop in the cooked waxy corn starch sample from 24 to 48 h is likely to be erroneous, as no gas (Fig. 1) or SCFAs (Fig. 3a) were generated during this time period. These data show that starch-entrapped microspheres ferment more slowly than the waxy corn starch control.

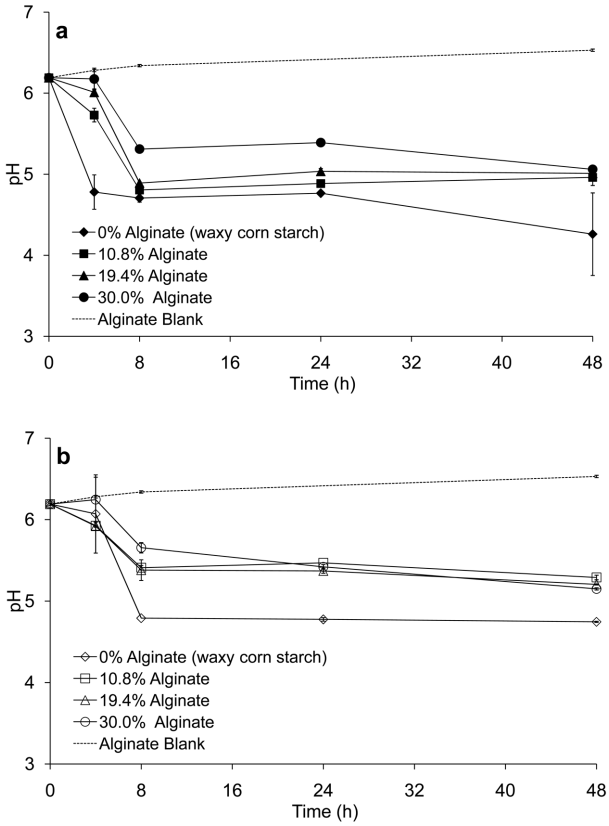

Figure 2. pH changes during *in vitro* fermentation of starch-entrapped 10.8, 19.4, and 30.0% alginate microspheres and waxy corn starch. Filled shaped are cooked samples (a); open shapes are uncooked samples (b). Error bars show SD; some error bars are too small to see; $n = 2$.

Uncooked samples (Fig. 2b) showed significant drops in pH between 4 and 24 h of fermentation ($p < 0.05$). Only the uncooked starch-entrapped 30.0% alginate microspheres caused a significant decrease in pH from 8 to 48 h of fermentation ($p < 0.05$). This would be due to the tighter alginate matrix as compared to the uncooked starch-entrapped 10.8 and 19.4% alginate microspheres. After 48 h of fermentation, the starch-entrapped microspheres showed similar pH values; however, waxy corn starch showed a lower

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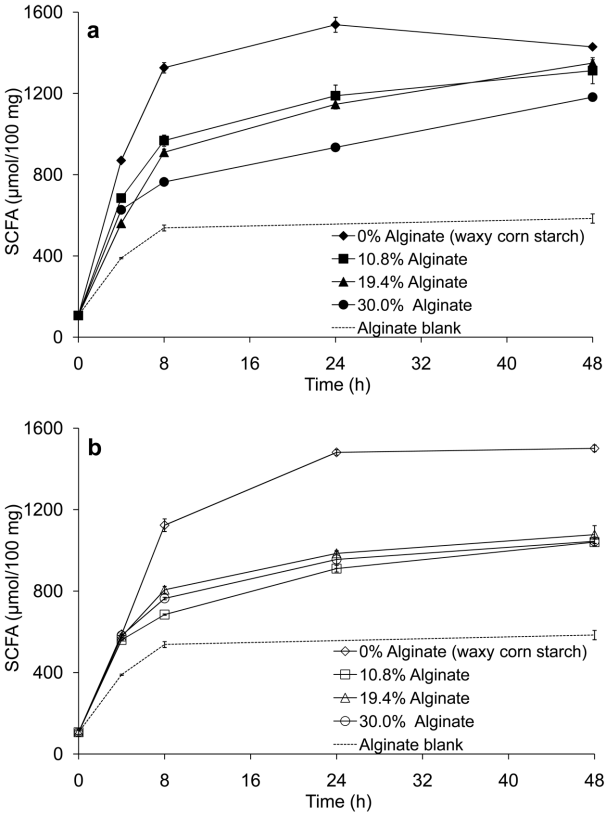

Figure 3. SCFA produced during *in vitro* fermentation of starch-entrapped 10.8, 19.4, and 30.0% alginate microspheres and waxy corn starch. Filled shaped are cooked samples (a); open shapes are uncooked samples (b). Error bars show SD; some error bars are too small to see; $n = 2$.

pH than all starch-entrapped microspheres. This indicates that the raw starch-entrapped microspheres may be incompletely fermented even after 48 h of fermentation.

3.3.4 Short chain fatty acid production

As expected, SCFA production correlated with the changes in pH during *in vitro* fermentation. In cooked samples (Fig. 3a), entrapment of starch resulted in significantly less SCFA production after 48 h of fermentation when compared to waxy corn starch ($p < 0.0001$). Notably, however, starch entrapment resulted in significant increases in SCFA production for all samples between 24 and 48 h of fermentation, whereas waxy corn starch showed a significant decrease ($p < 0.05$). Similar trends were found for uncooked samples (Fig. 3b), except a significant increase in SCFA production between 24 and 48 h of fermentation was only observed from the microbiota fed starch-entrapped 10.8% alginate microspheres ($p < 0.001$).

These are important findings because under physiological conditions, SCFAs are absorbed and utilized as they are produced. Therefore, slowly fermentable carbohydrates may allow bacteria to continually produce SCFAs during prolonged fermentation. Usually, the distal colon is chronically low in fermentable substrate and SCFAs [9], resulting in putrefactive fermentation and the production of undesirable, toxic compounds. This might be prevented by starch-entrapped microspheres.

Figure 3 also shows that cooking increased bacterial SCFA production compared to uncooked microspheres, except when the microbiota were exposed to starch-entrapped 30.0% alginate microspheres. This is a notable finding considering that the volume of gas produced by all starch-entrapped microspheres was not statistically different ($p > 0.05$), implying that cooking increases the production of SCFAs during fermentation without increasing the production of gas. Similarities in SCFA production between the uncooked and cooked starch-entrapped 30.0% alginate microspheres may be due to the high density of the matrix such that cooking did not cause sufficient swelling to change fermentation properties. Despite producing lower levels of SCFAs compared to cooked microspheres, the uncooked starch-entrapped microspheres still produced high amounts, rivaling the production reported previously for inulin [34], β-glucan [29], and resistant starch [19] using similar *in vitro* procedures (converted to equivalent units: µmol/100 mg substrate). Even though these studies cannot be directly compared, due to differences in bacterial populations among individuals [35], it shows that (un)cooked starch-entrapped microspheres are highly fermentable.

In general, starch causes proportionally more butyrate production than other polysaccharides during fermentation [36]. Indeed, regardless of whether the starch-entrapped microspheres were cooked or not, there was a production of between 200 and 300 µmol of butyrate per 100 mg substrate after 24 h of fermentation of starch-entrapped microspheres (data not shown). Others have reported ranges of 100–200 mol/100 mg for inulin [34] and β-glucan [29].

Table 2 shows the molar fractions of acetate, propionate, and butyrate produced after 48 h of fermentation. Few differences were observed in propionate ratios; however, the acetate fraction tended to decrease, and the butyrate fraction tended to increase with a higher percentage of alginate in the microspheres. This is not surprising, as acetate and butyrate metabolism are intimately connected [37]. This trend was more pronounced in the cooked samples, but still statistically significant in uncooked samples. The cooked starch-entrapped 30.0% alginate microspheres produced statistically similar molar fractions of butyrate (Table 2) compared to waxy corn starch, even though it was more slowly fermented.

3.3.5 Bacterial fingerprinting

To determine whether starch-entrapped microspheres have the ability to change the microbiota, we assessed the microbial fingerprint of the initial inoculum and for each sample after 48 h of fermentation using LH-PCR fingerprinting analysis. Figure 4 shows the normalized abundances for each amplicon length present at >1% of the total population

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Table 2. Molar fractions of SCFAs produced after 48 h of *in vitro* fermentation

Alginate (%)	Molar fraction		
	Acetate	Propionate	Butyrate
Cooked			
0[a)]	0.65^{D}	0.08^{ABC}	0.28^{AB}
10.8	0.76^{ABC}	0.05^{BC}	0.19^{C}
19.4	0.71^{CD}	0.06^{ABC}	0.23^{BC}
30.0	0.64^{ABC}	0.07^{ABC}	0.29^{A}
Uncooked			
0	0.75^{BC}	0.04^{C}	0.20^{C}
10.8	0.84^{A}	0.09^{AB}	0.07^{A}
19.4	0.81^{D}	0.08^{ABC}	0.11^{DE}
30.0	0.78^{ABC}	0.09^{A}	0.13^{D}

Values in the same column with the same superscript (capital letters) are not significantly different ($p > 0.05$). Because of rounding, some of the same numbers may have different superscripts. The alginate blank has been subtracted from these values. $n = 2$.

a) Waxy corn starch (control).

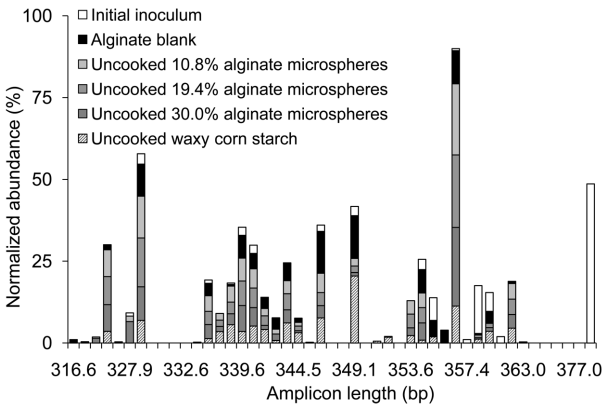

Figure 4. Stacked histogram of LH-PCR bacterial fingerprints of initial inoculum before fermentation and the alginate blank, uncooked starch-entrapped microspheres, and uncooked waxy corn starch after 48 h of fermentation. DNA from duplicate samples for each treatment was extracted and then four replicate fingerprints for each duplicate were analyzed.

for the initial inoculum prior to fermentation, and the microbiota receiving no carbohydrate substrate (alginate blank), uncooked starch-entrapped microspheres, and uncooked waxy corn starch after 48 h of fermentation. The lower 1% of the data were excluded to simplify the results, and because it was assumed that these populations have a minimal contribution to the gut metabolism as a whole.

There were clear differences between the initial inoculum and all fermented samples (Fig. 4); some OTUs were lost (OTUs 333.7, 357.4, 360.1, and 377.8), while others were gained (OTUs 336.9, 341.4, 342.5, 343.5, 344.5, 353.6, and 362.6); some OTUs were considerably diminished (OTUs 323.5 and 338.3), while others were substantially enhanced

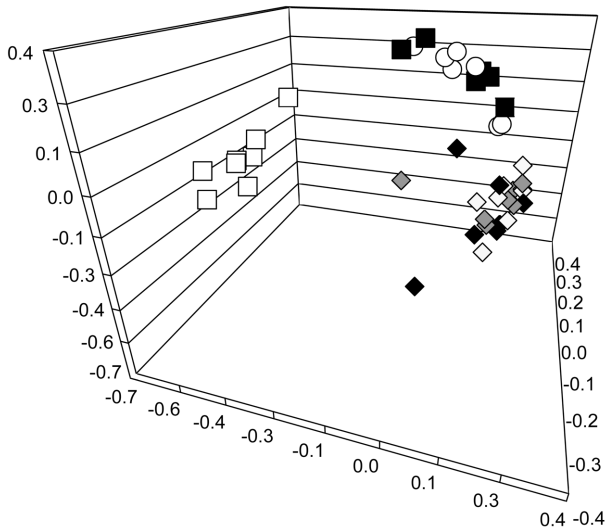

Figure 5. PCA of LH-PCR bacterial fingerprints of initial inoculum before fermentation (white squares) and the alginate blank (black squares), uncooked starch-entrapped microspheres (10.8% alginate, white diamonds; 19.4% alginate, gray diamonds; 30.0% alginate, black diamonds), and uncooked waxy corn starch (white circles) after 48 h of fermentation. DNA from duplicate samples for each treatment was extracted and then four replicate fingerprints for each duplicate were analyzed.

(OTUs 358.4 and 355.2). Comparing the microbiota receiving starch-entrapped microspheres to bacteria exposed to waxy corn starch, there was a disappearance of OTU 355.2, and an appearance of OTU 327.9; OTU 354.5 was diminished, while OTUs 349.1 and 351.1 were enhanced. Figure 4 also suggests that the no carbohydrate (alginate blank) and waxy corn starch had more similar effects on the microbiota than the starch-entrapped microspheres (see OTUs 323.5, 328.9, 334.4, 341.4, 343.5, and 359.4).

To illustrate the microbial shifts more clearly, we performed PCA using Bray Curtis similarity. Figure 5 shows the presence of a cluster representing the initial inoculum (white squares), which is clearly separated from the fermented samples. This indicates that there was a shift in microbial communities during fermentation, as expected. Microbiota receiving no carbohydrate (alginate blank; black squares) and those exposed to uncooked waxy corn starch (white circles) cluster together, indicating that the alginate blank and waxy corn starch had similar influences on the microbial shift during fermentation, when comparing initial time ($t = 0$ h) to the final time ($t = 48$ h). Surprisingly, the bacterial communities in all of the fermented uncooked starch-entrapped microsphere treatments clustered together, but were distinct from the initial inoculum, alginate blank, and the waxy corn starch samples, indicating that uncooked starch-entrapped microspheres had a unique effect on the microbial community during fermentation that persisted up to 48 h of fermentation.

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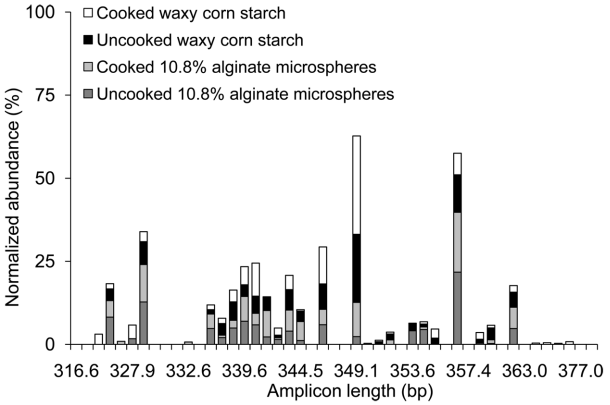

Figure 6. Stacked histogram of LH-PCR bacterial fingerprints of cooked and uncooked starch-entrapped 10.8% alginate microspheres, and cooked and uncooked waxy corn starch after 48 h of fermentation. DNA from duplicate samples for each treatment was extracted and then four replicate fingerprints for each duplicate were analyzed.

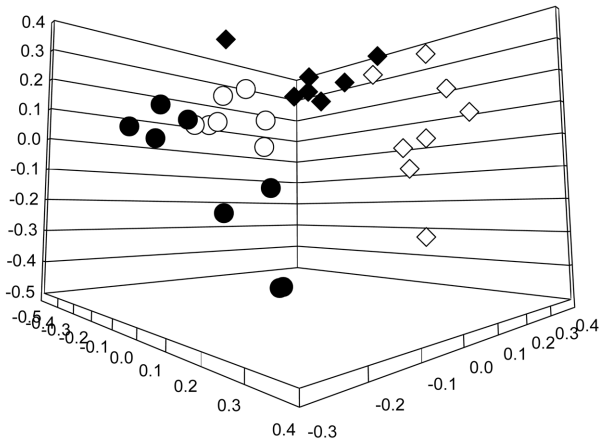

Figure 7. PCA of LH-PCR bacterial fingerprints of cooked (black diamonds) and uncooked (white diamonds) starch-entrapped 10.8% alginate microspheres, and cooked (black circles) and uncooked (white circles) waxy corn starch after 48 h of fermentation. DNA from duplicate samples for each treatment was extracted and then four replicate fingerprints for each duplicate were analyzed.

There are a number of reasons that might explain the differences in the observed microbial shifts. First, as to why the alginate blank and the waxy corn starch clustered together, a possible reason has to do with the rapid rate of waxy corn starch degradation. Data for gas (Fig. 1) and SCFA (Fig. 3) production indicate that waxy corn starch was completely utilized within the first 24 h of fermentation. During this period of time, the starch may have caused an initial microbial shift; however, during the second 24 h of fermentation, the microbiota could have shifted to a pattern similar to the alginate blank. Accordingly, the long fermenting starch-entrapped microspheres provided continual carbohydrate substrate to support the microbiota shift at 48 h. Additionally, the mechanism of the bacterial utilization of the starch-entrapped microspheres and waxy corn starch may be different. Starch degrading colonic bacteria, such as *Bacteroides thetaiotaomicron* [38] and bifidobacteria [39], digest starch by first binding tightly to the granule surface, ensuring that degradation is rapid and efficient. In the case of starch-entrapped microspheres, the physical alginate barrier likely prevents adequate binding or movement of the bacteria, thus either requiring these bacteria to change their degradation mechanism, or allowing the proliferation of other bacteria that can digest the starch in a different manner. Alternatively, the microbial shift may be due to the differences in availability of soluble nutrients, or surface area of the substrate to be degraded. With normal waxy corn starch, all of the starch is readily available for fermentation and there is an initial high concentration of maltose and glucose. This allows microorganisms with low affinity for these carbohydrates, but high maximum growth rate, to predominate. Lactic acidosis in cattle is a good example: *Streptococcus bovis*, which has a poor affinity for glucose and maltose but a high maximum growth rate with high substrate concentration, grows rapidly when cattle are rapidly switched from a forage diet to a high concentrate diet, whereas *S. bovis* is normally a minor component of the rumen microbiota [40]. With the starch-entrapped microspheres, the rate of availability, and thus rate of hydrolysis, is much lower and there is little chance of high concentration of soluble breakdown products, thus microbiota that have a high affinity for maltose and glucose would predominate.

The stacked histogram in Fig. 6 depicts the communal differences between microbiota receiving uncooked and cooked starch-entrapped 10.8% alginate microspheres and those exposed to cooked and uncooked waxy corn starch. It appears that cooking affects the microbial shift. In the microbiota fed starch-entrapped 10.8% alginate microspheres, OTUs 324.3, 332.6, and 351.1 are only present in the cooked samples, while OTUs 327.9 and 353.6 are only present in the uncooked samples. In the microbiota receiving waxy corn starch, OTUs 322.8, 327.9, and 375.8 are only present in the cooked samples, and OTU 341.4 is only present in the uncooked sample. It appears that the uncooked starch-entrapped 10.8% alginate microspheres may have unique effects on the microbial community compared to the other three samples shown in Fig. 6; there are three OTUs that are present in all other samples, but not in the uncooked starch-entrapped microspheres: OTUs 350.7, 351.1, and 358.4. Additionally, some OTUs are substantially diminished (OTU 359.4) or enhanced (OTUs 353.6 and 354.5) when comparing all other samples to the uncooked starch-entrapped 10.8% alginate microspheres.

PCA analysis of the uncooked *versus* cooked samples (Fig. 7) clearly shows that cooking the starch-entrapped microspheres shifts the microbial community towards the

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waxy corn starch cluster. This may be because the swelling caused by cooking allows the bacteria sufficient access to the starch within the alginate matrix such that the bacteria may ferment the starch in a manner similar to that of waxy corn starch. Notably, however, under physiological conditions, it is unlikely that a significant amount of waxy corn starch would reach the colon for fermentation by bacteria, while much of cooked starch-entrapped microspheres may survive digestion and be fermented.

4 Concluding remarks

Entrapment of starch slows the small intestinal *in vitro* digestion rate such that most of the starch remains intact for fermentation, thereby creating a controlled-release resistant starch. Advantages of entrapment compared to waxy corn starch are a reduction in the rate of gas production, and significant increases in both SCFA and butyrate production during the latter stages of fermentation (24 – 48 h). There was significantly less SCFA production from the microbiota exposed to starch-entrapped microspheres compared to waxy corn starch, although this difference was relatively small. Cooking increased the production of total SCFAs, including butyrate, except in samples with the highest percentage alginate (30.0%) in the microspheres. Increasing the percent alginate or cooking the microspheres increased the proportion of butyrate produced. The differences in fermentation SCFA profiles between starch-entrapped microspheres and waxy corn starch appear to be, at least in part, due to changes in fecal microbiota. Bacterial fingerprinting revealed that starch-entrapped microspheres have a unique effect on the microbiota that is different from waxy corn starch. Upon cooking, however, starch-entrapped microspheres show a microbial shift back toward that of waxy corn starch.

These results suggest that starch-entrapped microspheres may provide a beneficial source of dietary fiber with the following unique characteristics: (i) low rate of gas production; (ii) slow fermentation rate; and (iii) capability of influencing the microbial pattern. Further human studies are needed to determine whether these predictions actually occur *in vivo*.

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