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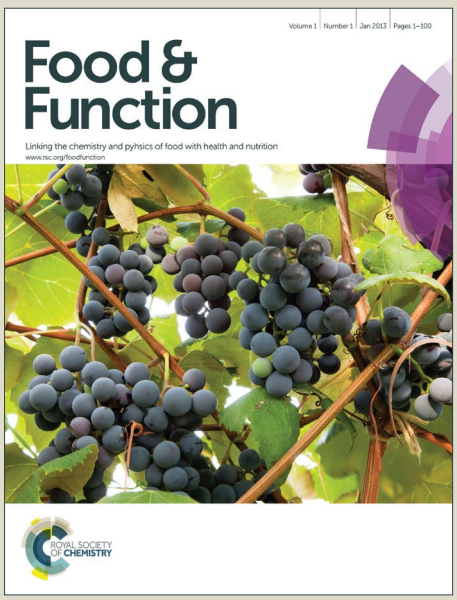

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In Vitro Analysis of Partially Hydrolyzed Guar Gum Fermentation Differences Between Six Individuals

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Abstract

Partially hydrolyzed guar gum (PHGG) is a fermentable, soluble, non-gelling fiber consumed as both a supplement and ingredient. PHGG supports bifidogenic and lactogenic growth, and increases the concentration of short chain fatty acids (SCFAs) in the distal intestine due to its fermentability. Changes in SCFA development due to the fermentation of dietary fibers in the colon have been widely studied, but there are limited studies analyzing the differences in SCFA development across multiple individuals (ages 23-68) exposed to the same dietary fiber (PHGG). With the six donors analyzed in this study, gas production varied from 59-80 mL/0.5g fiber at 12 h and 85-93 mL/0.5g fiber at 24 h between the six donors. At 12 h butyrate concentrations varied from 6.99 µmol/mL to 23.84 µmol/mL and from 8.78 µmol/mL to 22.84µmol/mL at 24 h. Total SCFA concentration at 24 h ranged from 42.85 µmol/mL to 91.17 µmol/mL. The overall average SCFA ratio for the six fecal donors was 30:45:25 (acetate:propionate:butyrate), which is similar to other fermentable fibers analyzed using in vitro systems. SCFA development in the distal intestine increases the amount of metabolizable energy from the diet, but varies greatly among people based primarily on the composition and changes of their gut microflora. With over a 2-fold difference in SCFA production, significant differences were found among healthy individuals fecal microflora when exposed to PHGG. Donor 6 SCFA concentrations decreased at 24 h, indicating a quicker fermentation process than the other five donors. All SCFAs measured fluctuated greatly among the six individuals within 24 h of analysis. Results of *in vitro* fermentation analyses are limited by the wide variation found with fecal donor.

Keywords: acetate, propionate, butyrate, SCFA.

Introduction

Dietary fiber consumption in the U.S. is approximately 17g/d for adults[1], far below the recommended values.[2] The health benefits of adequate fiber intake include the ability to help maintain a healthy body weight[3,4], improved cardiovascular health[5,6,7], digestive system health[8], and supporting beneficial growth of the gut microflora.[9] An under-researched area is individual variation of fermentation dynamics, depending largely on the composition of the host's gut

microflora. Many studies have demonstrated that changes in SCFA concentrations are primarily due to fluctuations in the host's bacterial makeup.[10–12]

Partially hydrolyzed guar gum (PHGG) is a commonly consumed fiber formed from the controlled hydrolysis of guar gum, and is composed of mannose and galactose monomers. PHGG has been shown in randomized, cross-over clinical studies to reduce hunger while increasing satiety.[13,14] PHGG has also been shown to increase levels of bifidobacteria and lactobacilli[15,16], two beneficial genera of bacteria. In a clinical feeding study, subjects that consumed 20g/d of PHGG for four weeks showed decreased total serum cholesterol, increased fecal weight and output frequency and lower fecal pH without influencing fat, protein or mineral absorption.[17] PHGG has also been shown to alleviate irritable bowel syndrome (IBS) due to its non-gelling capacity and therapeutic effects.[18]

Short-chain fatty acids (SCFA) are commonly measured end-products of colonic fermentation. SCFA can contribute between 1.5-2.5 kcal/g[19], contributing up to 10% of metabolizable energy (ME) to the diet. Schwiertz *et al* found that there was a higher concentration of SCFAs in overweight and obese individuals.[20] Similar studies have correlated higher ratios of *Firmicutes* to *Bacteroidetes* and increased concentrations of SCFAs with obesity.[21–23] This increase in metabolizable energy also has many other beneficial effects to the consumer.[24]

SCFAs can act as anti-diarrheal agents by their stimulation of water and sodium absorption in the distal intestine, which may be one of the reasons why diarrhea is sometimes a consequence of impaired fermentation in the distal intestine. Antibiotics sometimes cause diarrhea and have been shown to drastically decrease SCFA concentrations in vitro.[25] Concentrations and oxidation rates of SCFAs may also play on important role in the pathogenesis of colitis.[26]

The objective of this study was to compare SCFA development among six donor's fecal microflora after exposure to PHGG in an in vitro fermentation system, with the secondary measurement of total gas production to analyze the differences in fermentation rates within the first 24 h among six individuals. To our knowledge, this is the first study to analyze inter-individual fermentation differences among six individuals exposed to PHGG within 24 h.

Materials

Fiber analyzed in this study was partially hydrolyzed guar gum (Benefibra™, Novartis Consumer Health Spa Origgio, Varese, Lombardy, Italy). Chemical reagents used were provided by ThermoFisher Scientific (ThermoFisher Scientific Inc., Waltham, MN, USA), Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA) and Oxyrase (Oxyrase Inc., Mansfield, OH, USA).

Methods

Donor Information

Table 1. Demographic Characteristics of Six Fecal Donors.

	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6
Age	31	68	60	24	22	21
Sex	Male	Male	Female	Male	Male	Male
BMI	23.7	33.6	19.5	26.3	24.7	23.0

Fecal Collection

Fecal samples were collected from six healthy volunteers (5 males, 1 female) under anaerobic conditions from individuals (ages 21-68) consuming non-specific Western diets, free of any antibiotic treatments in the last year, not affected by any GI diseases and not consuming any probiotic or prebiotic supplements. Fecal samples were anaerobically collected within 30 minutes of the start of the fermentation, and homogenized immediately upon collection. All data and samples collected were done in accordance with University of Minnesota policies and procedures.

Fermentation

Fiber samples (0.5 g) were hydrated in 40 mL of prepared sterile tricase peptone fermentation media in 100 mL serum bottles, capped, and incubated for 12 hours at 4°C. Following incubation, serum bottles were transferred to a circulating water bath at 37°C and allowed to incubate for 2 hours. Post-collection, fecal samples were mixed using a 6:1 ratio of phosphate buffer solution to fecal sample. After mixing, obtained fecal slurry was combined with prepared reducing solution (2.52 g cysteine hydrochloride, 16 mL 1N NaOH, 2.56 g sodium sulfide nonanhydride, 380 mL DD H_2O) at a 2:15 ratio. 10 mL of the prepared fecal inoculum was added to each of the serum bottles, 0.8 mL Oxyrase® was added, flushed with CO_2, sealed, and then immediately placed in a 37°C circulating water bath. Samples were prepared in triplicate and analyzed at 0, 12 and 24 h. Upon removal at each time point, total gas volume was measured. Then, 1 mL of copper sulfate (200 g/L) was added to cease fermentation. Lastly, 2 mL aliquots were frozen at -80°C for SCFA analysis.

Gas Analysis

Total gas production was measured by syringe difference analysis. Gas was measured by piercing cap of serum bottle with syringe needle and measuring gas released from each individual sealed serum bottle.

SCFA Analysis

SCFA extraction methods were adapted and slightly modified from Schneider *et al*.[27] 2 mL aliquots were removed from the -80°C freezer and placed in a 4°C cooler for 12 hours prior to analysis. Tubes were then gently vortexed for 5 seconds. Then, 1.6 mL of DI H_20, 400µL H_2SO_4 (50% vol/vol), and 2 mL diethyl ether (premixed with 2-ethyl butyric acid as internal standard) were all added to tubes and vortexed again for 5 seconds. Tubes were then placed in an orbital

shaker for 45 minutes at 100 RPM. Tubes were removed and then centrifuged for 5 minutes at 3000 RPM. The supernatant was removed from tube and placed in 10 mL tubes containing $CaCl_2$ to remove residual water. The solution was then filtered using a BD 1 mL syringe (Becton, Dickinson and Company Franklin Lakes, NJ) and a Millex 13 mm nylon membrane filter with a 0.20 µm pore size (Merck Millipore Ltd Tullagreen, Carrigtwohill, Co. Cork, IRL). Extractions were then analyzed using a HP 5890 series gas chromatograph (Hewlett Packard, Palo Alto, CA) with a 30 m x 0.250 mm x 0.25 µm polyethylene glycol (PEG) column (Agilent Technologies, USA), with a 110°C oven temperature. Samples were injected using an automated HP 7673 GC/SFC injector (Hewlett Packard, Palo Alto, CA). Injector and detector temperatures were 220°C and 240°C, respectively. Flow rates for air, helium and hydrogen were 26, 28 and 315 mL/min, respectively. All samples were analyzed utilizing a 50:1 split ratio.

Statistical Analysis

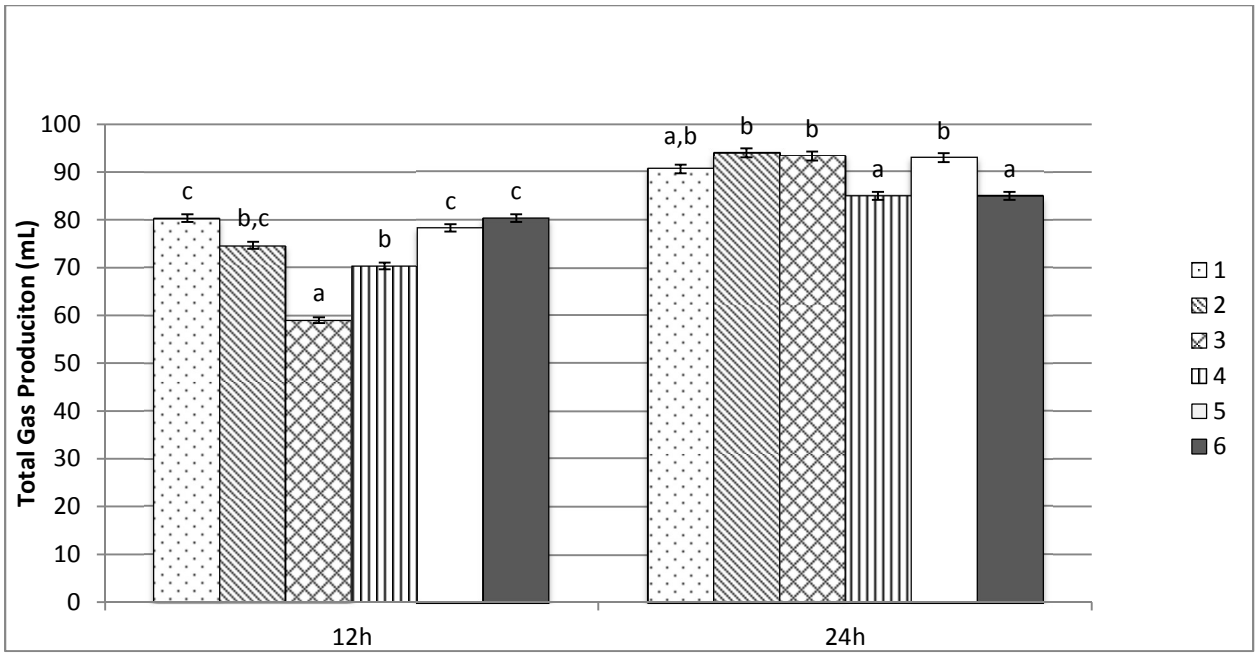
All statistical analysis was conducted using SPSS (SPSS Chicago, IL). Analysis of variance (ANOVA) with Tukey HSD was used for all tests measuring differences among means. Log transformations were applied where necessary. Statistical significance was achieved for p-values less than 0.05.

Results

Gas Production

At 12 h post-inoculation, gas production ranged from 59 mL to 80 mL (Table 1), with an overall average production of 74 mL, similar to previously published data.[15] At 24 h, gas production ranged from 85 mL to 93 mL, with an overall average gas production of 90.2 mL for the six individuals. Between 12 h and 24 h of analysis the average increase in gas production was 16.3 mL, but ranged between 5 mL to 34 mL increases, with all individuals having higher gas production at 24 h compared to 12 h.

Figure 1. Total Gas Production Comparing Differences Among Six Individuals at 12 h and 24 h Post-Exposure to PHGG.



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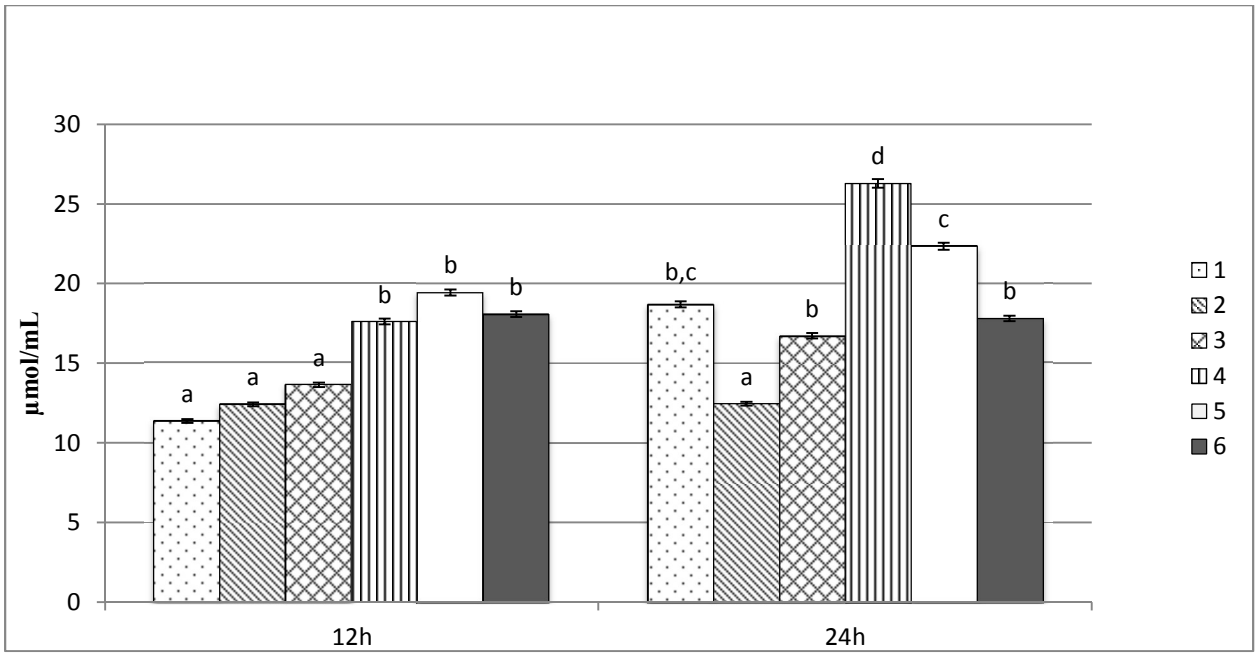
130 * Values displayed are means (n=3) ± SE for each individual at 12 h and 24 h.

131 * Columns with different letters are significantly different from one another within each time of
132 measurement. Histograms with data were analyzed using ANOVA with Tukey HSD ($p<0.05$).

133 *SCFA Production*

134 Acetate production varied greatly among the six donors, with concentrations increasing at 24 h
135 compared to 12 h for 5 of the six donors (Figure 2). Donor 4 had similar concentrations to donor
136 5 and 6, $p=0.343$ and $p=0.803$, respectively, but had the highest concentration at 24 h. Although
137 donors 4, 5 and 6 had similar concentrations at 12 h, they were all statistically different at 24 h (4
138 vs. 5, $p=0.047$; 4 vs. 6, $p<0.001$; 5 vs. 6, $p=0.024$). At 12 h, donors 1,2 and 3 had similar
139 concentrations (1 vs. 2, $p=0.580$; 1 vs. 3, $p=0.239$; 2 vs. 3, $p=0.524$), and at 24 h boths donor 1
140 and 3 had similar concentrations ($p=0.305$), while donor 2 was significantly lower than both (1
141 vs. 2, $p=0.003$; 2 vs. 3, $p=0.033$).

142 Figure 2. Acetate Production at 12 h and 24 h of Fermentation of PHGG by Six Individuals.



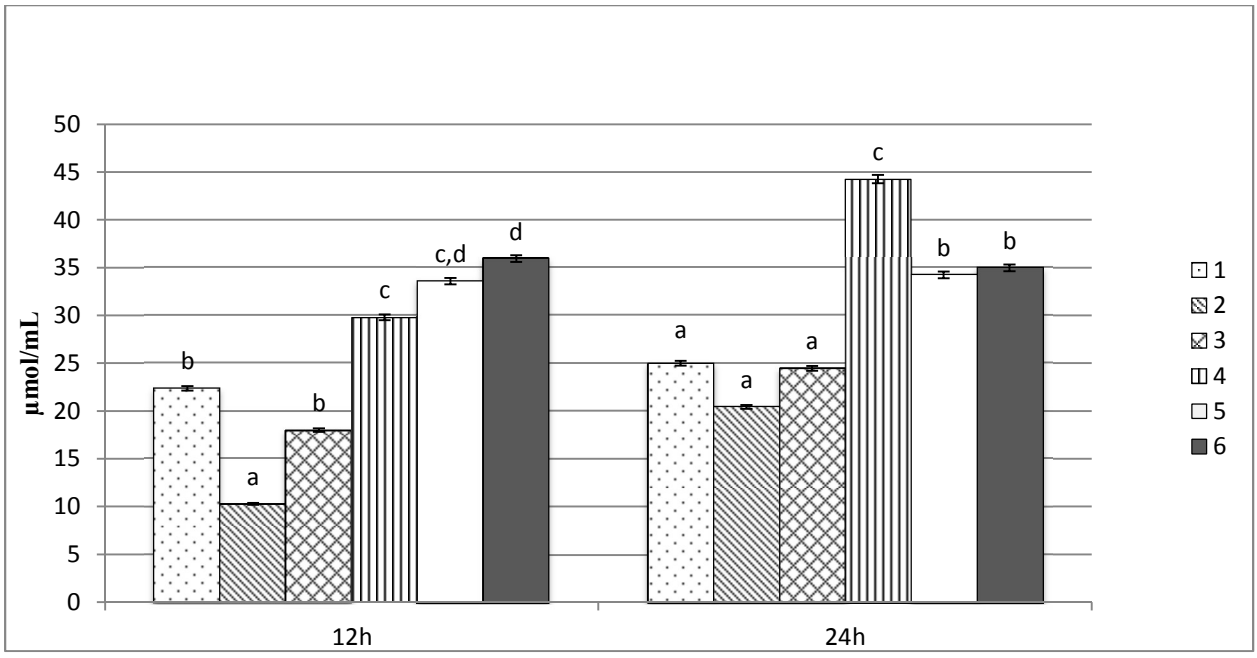
143

144 * Values displayed are means (n=3) ± SE for each individual at 12 h and 24 h.

145 * Columns with different letters are significantly different from one another within each time of
146 measurement. Histograms with data were analyzed using ANOVA with Tukey HSD ($p<0.05$).

147 Propionate concentrations (Figure 3) closely resemble the acetate concentrations (Figure 2) in
148 that donors 1, 2 and 3 had the lowest concentrations at 12 and 24 h, and donor 4 had the highest
149 concentration at 24 h. At 12 h donor 2 had the lowest concentration (2 vs. 1, $p<0.001$; 2 vs. 3,
150 $p=0.012$). Donor 2 also had the lowest concentration at 24 h, but statistically similar to donor 1
151 and donor 3, $p=0.115$ and $p=0.161$, respectively. At 24 h of exposure, donor 4 had the highest
152 concentration (4 vs. 5, $p=0.001$; 4 vs. 6, $p=0.003$).

153 Figure 3. Propionate Production at 12 h and 24 h of Fermentation ofPHGG by Six Individuals.



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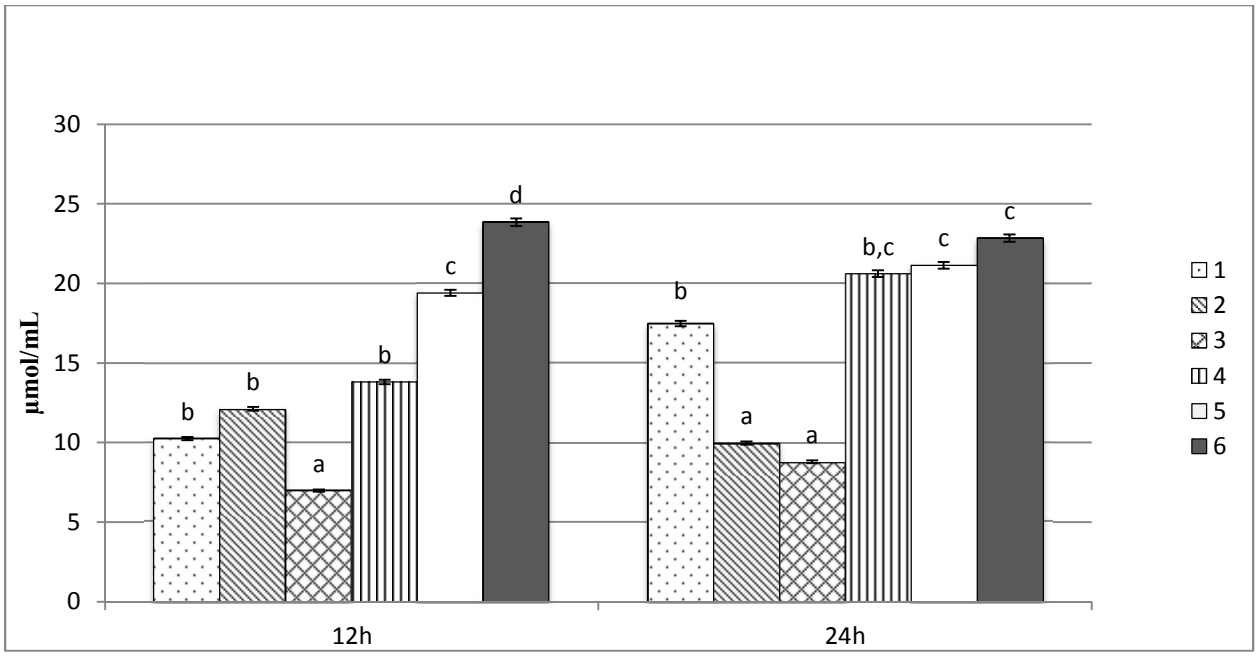
155 * Values displayed are means (n=3) ± SE for each individual at 12 h and 24 h.

156 * Columns with different letters are significantly different from one another within each time of
157 measurement. Histograms with data were analyzed using ANOVA with Tukey HSD ($p<0.05$).

158 Butyrate concentrations and changes in concentrations varied greatly among the six donors
159 (Figure 4). Donor 3 had the lowest butyrate concentration at 12 h (3 vs. 1, $p=0.048$; 3 vs. 2,
160 $p=0.003$), and at 24 had statistically similar concentration compared to donor 2 ($p=0.455$) and a
161 lower concentration than donor 1 ($p<0.001$). Donor 6 had the highest concentration at 12 h (6 vs
162 5, $p=0.009$) and had similar concentrations to donors 4 and 5 at 24 h (6 vs. 5, $p=0.288$; 6 vs. 4,
163 $p=0.166$).

164 Figure 4. Butyrate Production at 12 h and 24 h of Fermentation of PHGG by Six Individuals.



* Values displayed are means (n=3) ± SE for each individual at 12 h and 24 h.

* Columns with different letters are significantly different from one another within each time of measurement. Histograms with data were analyzed using ANOVA with Tukey HSD ($p<0.05$).

Between Donor 4 and Donor 2 at 24 h of exposure there was over a 2-fold difference in total SCFA production (Table 2). Donor 6 was the only subject to have a decrease in total SCFA at 24 h compared to 12 h. This is likely due to quicker ability to ferment PHGG prior to the 12 h measurement. Donor 5 had similar concentrations to donor 6 ($p=0.352$) at 12 h and had similar concentrations to donor 6 ($p=0.717$) at 24 h, but less than donor 4 ($p=0.028$).

Table 2. Average Total SCFA Profiles (µmol/mL) for Six Donors at 12 h and 24 h Post-Exposure to PHGG Treatment.

Donor	12 h	24 h
1	43.98(6.21)^{a}	61.17(4.81)^{b}
2	34.84(0.88)^{a}	42.85(4.71)^{a}
3	38.57(2.53)^{a}	49.97(3.54)^{a,b}
4	61.23(4.34)^{b}	91.17(4.47)^{d}
5	72.43(3.47)^{b,c}	77.73(4.32)^{c}
6	77.89(2.37)^{c}	75.62(4.36)^{c}

*Values are means of triplicate determinations (SEM). Means within columns with different letters are significantly different from one another. Data were analyzed using ANOVA with Tukey HSD ($p<0.05$).

*Total SCFA include: acetate, propionate and butyrate.

Discussion

In vivo, SCFA production by humans is usually between 100-200 mM per day, but is highly dependent on the host's environment and availability of substrate for fermentation.[28] The average total SCFA concentration after 24 h of analysis was 60.3 mM/L for all six fecal donors in this study. Once produced, over 95% of all SCFAs are immediately absorbed, often making them hard to accurately measure in vivo. The three most abundant SCFAs (acetate, propionate and butyrate) are commonly formed due to the fermentation of non-digestible carbohydrates and proteins. Other acids that escape digestion are typically formed due to the breakdown of branched-chain amino acids that surpass digestion in the upper gastrointestinal tract typically include: valerate, isovalerate, isobutyrate, 2-methyl-butyrate, formate and caproate.[29] Acetate is primarily metabolized for energy in the muscles[30], propionate used as a gluconeogenic substrate outside of the colon[31], and butyrate as a fuel for colonocytes.[32] Typical ratios for acetate:propionate:butyrate range from 40:40:20 to 75:15:10, depending on substrate that is available for colonic fermentation.[29,33] Many studies show that the order of concentration typically follows acetate > propionate > butyrate, but actual concentrations vary between studies depending on study design[17,34,35]. The average approximate ratio for this study was 30:45:25, but varied greatly among the six fecal donors. Although it is well accepted that PHGG is extensively fermented in the gut, little data on SCFA production with PHGG have been published.

Many studies have analyzed the impact of different fibers and other macronutrients and how they affect SCFA production in many in vitro models[36–38], but to our knowledge, this is the first that addresses differences among six individuals within 24 h of exposure. One of the first in vitro studies to analyze differences for both inter-individual and intra-individual relationships between SCFA ratios was conducted by Mortensen et al.[39], and showed that there was a significant correlation between substrate analyzed and resulting SCFA production, and no significant differences in inter-individual or intra-individual comparisons with the three similar donors used in the study. However, six drastically different substrates (glucose, wheat bran, pectin, ispaghula, cellulose and albumin) were analyzed with only three fecal donors.

Total gas production measures gas produced during fermentation, primarily composed of CO_2, H_2, and CH_4.[40] Previous studies have shown that breath hydrogen and methane poorly represent fiber digestion.[41] Total gas production potentially indicates overall fermentation rates likely to be seen in the gut. Excessive gas production may lead to undesirable flatus, abdominal pain and bloating.

Overall, the SCFA profiles for each of the six donors were quite different at 12 and 24 h. With an average overall ratio of 30:45:25 (acetate:propionate:butyrate), acetate production was slightly less compared to other fermentable fibers in similar in vitro models.[42] With over a 2-fold change in total SCFA among donors, ratios fluctuated greatly among individuals. Donor 6 had concentrations of acetate, propionate and butyrate that were lower at 24 h than 12 h for each respective SCFA, and was the only donor to have decreased levels for multiple SCFA. With the

highest concentrations at 12 h of propionate and butyrate, and the second highest acetate concentration at 12 h it is clear that the PHGG was fermented primarily before the 12 h measurement. Compared to the two other males with similar ages and BMI (donors 4 and 5), differences in fermentation rates are likely due to differences in the fecal microflora.

In conclusion, the overall average SCFA ratio for the six fecal donors was 30:45:25 (acetate:propionate:butyrate), which is similar to other fermentable fibers analyzed using in vitro systems. At 24 h there was over a 2-fold difference among individuals, indicating significant differences among different individuals exposed to PHGG. With one donor displaying decreased concentrations of all SCFA at 24 h compared to 12 h, fecal microbiota from select individuals ferment the digestible components of PHGG completely within the first 12 h of exposure. Further studies should quantify those bacteria that ferment PHGG quicker than others, and correlations between SCFA concentration and targeted gut microbiota should be established. This work is ongoing in our laboratory and we plan to extend our *in vitro* studies to determination of changes in microbiota and whether these correlate to changes seen in SCFAs.

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