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Proceeding Paper

AG1, A Novel Synbiotic, Demonstrates Capability to Enhance Fermentation Using the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®) †

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Abstract: Synbiotics, a combination of prebiotics and probiotics, are growing in popularity with consumers desiring improved gastrointestinal health. Prebiotics are non-digestible nutrients that can be metabolized by microbiota to exert a beneficial effect while probiotics are live microorganisms themselves that can also exert beneficial effects when consumed. Due to the rise in prebiotic and probiotic usage, there has been concern from some experts that not all synbiotics indicated for use as nutritional supplements are properly evaluated for their biological efficacy. AG1 is a novel foundational nutrition supplement that has been designed to exert a synbiotic effect. In its formulation, AG1 contains traditional prebiotics, phytonutrients from wholefood sources and botanical extracts, and two probiotics (*Lactobacillus acidophilus* UALa-01 and *Bifidobacterium bifidum* UABb-10). Alongside ingredient evidence that AG1 exerts synbiotic effects, efficacy testing was performed using the Simulator of Human Intestinal Microbial Ecosystem (SHIME®) model. Physical and metabolic evidence of fermentation were used to evaluate the success of AG1 as a synbiotic. Data from the SHIME® model showed a significant increase ($p < 0.01$) in the total amount of short chain fatty acids (SCFAs), specifically with significant increases on total acetate ($p < 0.001$) and propionate ($p < 0.0001$) production, as well as gas production. These results were expected as both SCFAs and gas are the major byproducts of bacterial carbohydrate fermentation. These data suggest that AG1 exerts preclinical evidence of a synbiotic effect by human microbiota.

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1. Introduction

Supporting the diversity of the gut microbiome is a critical objective many nutritional supplements wish to confer. Increased species diversity tends to correlate with increased functionality in the human gut microbiome [1]. Some nutritional supplements like vitamins, prebiotics, and probiotics can either increase or reinforce gut microbiome diversity [2–4]. The most used supplements for supporting the health of the gut microbiome are prebiotics, probiotics, and their combination as synbiotics. Simply, prebiotics are nutrients that are degraded by gut microbiota [5], while probiotics are live microorganisms that when consumed in sufficient amounts confer health benefits [6]. When both are taken in adequate amounts, their respective health benefits are enhanced relative to when they are

taken alone indicating a synergistic relationship [7]. However, there is variability in how humans respond to synbiotics [8,9]. In many cases, this variability can occlude positive findings in clinical studies [9].

While it is virtually impossible to eliminate interpersonal variation in clinical studies, it is possible to account for and reduce variation by utilizing tightly controlled in vitro or ex vivo models when exploring the effect of synbiotic treatments in humans. The Simulator of the Human Intestinal Microbial Ecosystem (SHIME®), commonly referred to as just the SHIME model, was developed in 1993 [10]. The SHIME model had taken previous simulator models that failed to accurately capture heterogeneity intestinal microenvironment and created a multi-reactor system that could simulate the different physiological compartments of the colon [11]. Taking this into consideration, the ability to seed the reactors with donor stool from human subjects allows for unique and physiological simulations of the colonic microbiota and the evaluation of donor specific responses in a controlled environment [11]. Therefore, the SHIME model, and other models employing similar physiological conditions, could sufficiently and reproducibly, be used to evaluate stool from a diverse range of humans to capture preclinical data that can be used to sufficiently power clinical studies.

This current study sought to evaluate the fermentative capability of AG1® as a novel foundational nutrition synbiotic. As a synbiotic, AG1 contains two probiotics, *Lactobacillus acidophilus* UALa-01 and *Bifidobacterium bifidum* UABb-10, as well as prebiotics in the form of fiber (e.g., inulin) as well as phytochemicals with glycosidic residues (e.g., phenolic acids and flavonoids). While there is growing evidence that phytochemicals can act as prebiotics [12,13], the specific phytochemicals in AG1 have not been fully explored for their fermentative capacity. The primary objective of this study was to determine if AG1 could be fermentable by measuring byproducts of fermentation. Since specific microbiome communities have unique metabolic profiles, our second objective was to use the SHIME model to evaluate whether donor specific microbiomes would have donor specific effects on the overall fermentation of AG1.

2. Materials and Methods

2.1. Test Products

AG1® (AG1; Athletic Greens International, Carson City, NV, USA) is a novel foundational nutrition supplement containing a mixture of vitamins, minerals, prebiotics, probiotics, and phytonutrients. While 1 serving of AG1 is 12 g, each bioreactor received a dose of 6 g, due to the volume limitations of the simulator. The placebo group received only the blank control medium used to deliver AG1.

2.2. Test Gastrointestinal Tract System

We employed the SHIME® model adapted from Molly et al., 1993 which represented the stomach, small intestine, and proximal colon [10]. The model is comprised of two bioreactors to emulate the physiological conditions of the gastrointestinal tract. The first bioreactor was used to ensure the physiological conditions of the stomach and small intestines. The second bioreactor was used to ensure the physiological conditions of the proximal colon and housed the simulated human gut microbiome environment. Fasted conditions were simulated and maintained by adding a specific gastric suspension to and a standardized bile acid/enzyme solution. Specific pH conditions and incubation times were used to emulate in vivo conditions representative of each compartment of the human gastrointestinal tract.

2.3. Gastric Phase and Small Intestine Phase

The 6g dose of AG1 was incubated at 37° Celsius for 45 min under constant mixing via stirring. The gastric environment was held at a pH of 2.0. Pepsin and phosphatidylcholine (1000 U/mL and 0.02 mM, respectively) were added. The background medium

used contained only salts and mucins recommended by the consensus method with NaCl and KCl reaching concentrations of ~50 mM and ~7mM, respectively.

Following the gastric phase, the contents were mixed via stirring and pH was automatically increased from 2.0 to 6.5. Mixing occurred for 27 min at a constant pH of 6.5 to represent the duodenal phase. The jejunal and ileal phases were 3 h in total. A steady pH of 7.0 and temperature at 37 °C were maintained. To remove the digested fraction, a simulated absorptive process using a dialysis approach was employed. This method used a cellulose membrane with a cut-off of 14 kDa. The entire luminal content was transferred into the dialysis membrane and submerged in dialysis fluid with the solution being refreshed every hour. The pancreatic enzymes used during the small intestine phase included pancreatin and contained all the relevant enzymes. The activity was set at 1.12 TAME U/mL. Defined ratios of specific enzymes were used with the activity set at 3.1 TAME U/mL for trypsin and 0.76 BTEE U/mL for chymotrypsin. The bile salts used during the small intestine phase are derived from bovine bile. Following the 3-h small intestine phase the luminal content was collected and the undigested fraction was used to initiate the short-term colonic batch simulations.

2.4. Short-Term Colonic Batch Simulations

The short-term colonic incubations were conducted using colonic medium, fecal inoculum, and the luminal content from the small intestine phase. The colonic medium contained host- and diet-derived substances such as peptone, yeast extract, and L-cysteine. This colonic medium was mixed with the luminal content as metabolic input for the microbial fermentation. A 7 mL amount of fresh fecal matter from three healthy adults was used. The donors utilized in this current study were all considered to be healthy following ProDigest's criteria for being a "healthy" donor. The bioreactors were made anaerobic by flushing with nitrogen gas and were incubated for 2 days at 37 °C under shaking conditions. Gas pressure was measured initially and at the end of the 48 h. The net change in gas pressure was used to determine the production of gas as a byproduct of microbial fermentation. The local pH was determined at the end of the 48 h. Quantitative analysis of the short chain fatty acids was performed with capillary gas chromatography and coupled with a flame ionization detector. Isolation of the short chain fatty acids was performed using liquid-liquid extraction. Short chain fatty acid concentrations were determined after the 48 h.

2.5. Statistics

All statistics and subsequent graphs were performed using GraphPad Prism (version 10.0.0 for Windows, GraphPad Software, Boston, MA, USA, www.graphpad.com). Normality was assessed for each variable before running the appropriate statistical test using Q-Q plots. No instances of overt non-normality were noted and thus normality was assumed for each variable. To determine the extent of fermentation, paired t-tests were conducted on the total measured variables after 48 h. To identify donor specific effects, a simple one-way ANOVA was employed for each variable. Multiple comparisons tests were conducted using the Tukey's multiple comparisons test with adjusted *p*-values. *p*-values were recorded and reported, with *p*-values less than 0.05 as the threshold for significance.

3. Results

Data suggested that the addition of the non-digested fraction of AG1 increased the extent of fermentation (Figure 1). Relative to the blank control, there was a significant decrease in the local pH ($p = 0.003$) as well as a significant increase in gas pressure ($p = 0.002$). To ensure these changes were a result of carbohydrate fermentation, the level of short chain fatty acids was also quantified. There was a significant increase in total concentration of short chain fatty acids ($p = 0.002$). Of the short chain fatty acids, there were

also significant increases in the concentration of acetate (p = 0.001) and propionate (p = 0.0001), but not butyrate (p = 0.451).

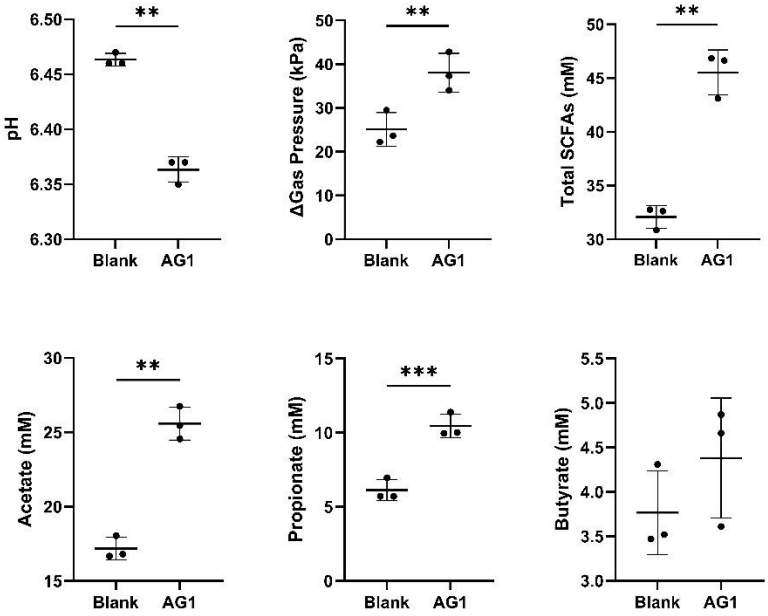

Figure 1. The metabolic byproducts of microbial carbohydrate fermentation were measured for AG1 treated and non-treated human gut microbiota after 48 h. Statistical analysis included paired t-tests. Data are shown as mean and standard deviation. ** $p < 0.01$; *** $p < 0.001$.

To understand the donor effect on fermentation capacity of AG1, the data from the biological replicates treated with AG1 were analyzed (Figure 2). Data showed that there was no significant difference in the local pH among the donor stool samples (p = 0.184). However, there was a significant difference in gas production among the donors (p = 0.003). The total concentration of short chain fatty acids also was significantly different (p = 0.004) with significance observed for acetate (p = 0.0003), propionate (p = 0.0008), and butyrate (p = 0.0006) among the donors. To further understand the differences between the individual donors, multiple comparisons tests were employed for each variable. In gas production, there was no significant difference between donor A and donor B, but a significant difference between donor B and donor C (p = 0.023) and between donor A and donor C (p = 0.002). For total SCFA production, there was no significant difference between donor A and donor B, but a significant difference between donor B and donor C (p = 0.008) and between donor A and donor C (p = 0.006). For acetate, there was a significant difference between donor A and donor B (p = 0.0002), a significant difference between donor B and donor C (p = 0.004), and between donor A and donor C (p = 0.02). For propionate, there was a significant difference between donor A and donor B (p = 0.001) and a significant difference between donor A and donor C (p = 0.001), but not between donor B and donor C. For butyrate, there was no significant difference between donor A and donor B, but a significant difference between donor B and donor C (p = 0.002) and between donor A and donor C (p = 0.0007).

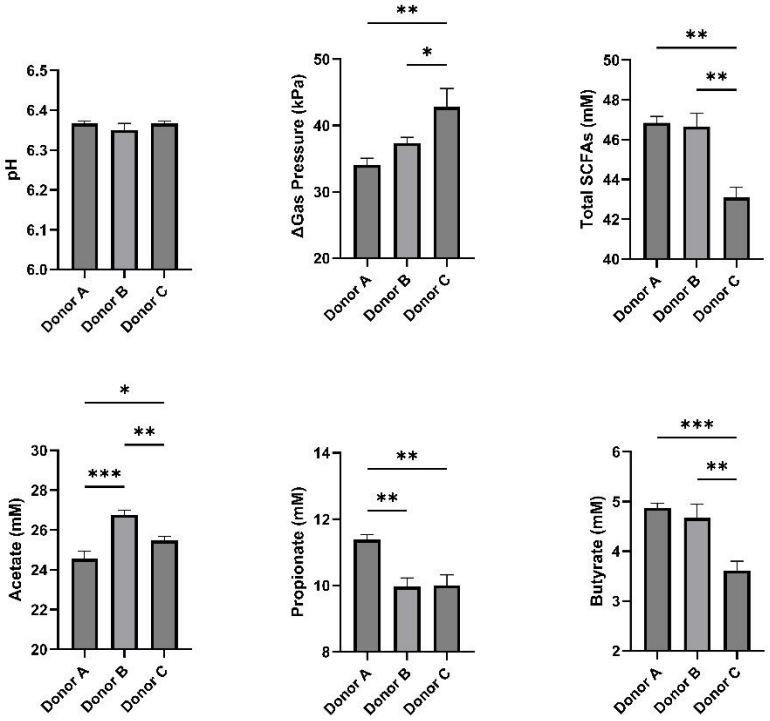

Figure 2. Donor specific differences in the metabolic byproducts of microbial carbohydrate fermentation were analyzed for AG1 treated human gut microbiota after 48 h. Statistical analysis included one-way ANOVA and Tukey's multiple comparisons tests. Data are shown as mean and standard deviation. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4. Discussion

There was sufficient evidence to suggest that AG1 was able to be fermented in this SHIME model resulting in significant SCFA production. Moreover, there was also information to suggest a donor specific fermentation effect. The results of this study are interesting when taken with the notion that there is no overt functional redundancy in the gut microbiome [14]. While there is notable functional diversity in dysbiotic or diseased microbiome states [15], there is a general misconception that there is functional redundancy in a healthy microbiome. This misconception stems from metagenomic profiling data which excludes non-mapped functions [16]. Because mapping is highly reliant on well characterized genes, anything that is not well understood is typically left out of these databases resulting in an artificial simplification of the microbiome's function. Some critical metabolic functions (e.g., oxalate and resistant starch degradation) are carried out by underrepresented taxa or rare species [17,18]. From the current data, we see significant differences in the metabolic output of carbohydrate fermentation from three seemingly healthy donors. This may partially begin to explain why there are mixed results from many prebiotic and probiotic clinical trials, there is significant functional deviations even in healthy donor microbiomes [19]. Perhaps underestimating the functional diversity in the human gut microbiome leads to underpowered clinical studies that yield mixed results pertaining to efficacy.

Based on the data from this experiment, there is evidence that AG1 can be fermented indicated by the increase in SCFA production and other byproducts of fermentation. While AG1 contains the prebiotic inulin, the observed fermentation is also likely attributed to the diverse composition of phytochemicals found in AG1. As mentioned, there is growing evidence that phytochemicals can act as prebiotics [12,13]. This is largely due to the fact that many phytochemicals, particularly phenolic acids and flavonoids, can have one or more carbohydrate residues bound to their hydroxyl groups (i.e., glycosides) [20].

These glycosides typically cannot be cleaved from the phytochemical by human enzymes or physical conditions during digestion. However, many bacteria in the gut do contain the proper enzymes to cleave these glycosides and utilize them for carbohydrate fermentation [21]. When this is considered in tangent to the fact that phytochemicals can alter microbiome composition [22], it becomes compelling to consider phytochemicals as prebiotics based on the definition of what a prebiotic is.

Because AG1 contains phytochemicals, has two probiotic species, and when used in the SHIME model produced evidence of increased carbohydrate fermentation, it is reasonable to conclude that there is preclinical data supporting the synbiotic potential of AG1. Further, while there were apparent donor specific effects on the metabolic output of the carbohydrate fermentation, there was still a robust and overall increase in fermentation. Despite these findings, it is important to note that only three donors were included in this study and only three biological replicates were obtained per donor. A small sample size (n = 3) is a limitation to these findings and must be expanded upon in subsequent experimentation. Taking this into consideration, care is needed when powering clinical studies on AG1 to account for individual microbiome differences and how they alter the nutritional supplement effect of AG1.

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Conflicts of Interest:

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