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The Novel Synbiotic, AG1®, Increases Short-Chained Fatty Acid Production in the Simulator of Human Intestinal Microbial Ecosystem (SHIME) Model®

Trevor O. Kirby [1,*], Jeremy R. Townsend [1,2], Philip A. Sapp [1], Marlies Govaert [3], Cindy Duysburgh [3], Tess M. Marshall [1], Massimo Marzorati [3,4] and Ralph Esposito [1,5]

1 Research, Nutrition, and Innovation, Athletic Greens International, Carson City, NV 89701, USA
2 Health & Human Performance, Concordia University Chicago, River Forest, IL 60305, USA
3 ProDigest BV, B-9052 Ghent, Belgium
4 Center of Microbial Ecology and Technology (CMET), Ghent University, B-9000 Ghent, Belgium
5 Department of Nutrition, Food Studies and Public Health, New York University-Steinhardt, New York, NY 10003, USA
* Correspondence: trevor.kirby@athleticgreens.com

Abstract: Recently, there is growing usage of prebiotics and probiotics as dietary supplements due to their purported health benefits. AG1® (AG1) is a novel foundational nutrition supplement which contains vitamins, minerals, phytonutrients, wholefood concentrates, adaptogens, and functional mushrooms. AG1 could be classified as a synbiotic because it contains traditional and non-traditional prebiotics (e.g., inulin and phytonutrients) as well as lactic-acid-producing probiotics. The purpose of this study was to employ the Simulator of Human Intestinal Microbial Ecosystem (SHIME®) model, which measures various aspects of gastrointestinal fermentation, to investigate the synbiotic effects of AG1. The SHIME experiment quantified gas production, changes in pH, and byproducts of carbohydrate and protein fermentation at baseline, 1, 24, and 48 h following the administration of AG1 or a blank control. The results indicated that AG1 significantly increased ($p < 0.05$; 41.9% increase) the production of total short-chain fatty acids (SCFAs) including acetate ($p = 0.001$; 49.0% increase) and propionate ($p < 0.001$; 70.8% increase). Regarding non-carbohydrate fermentation byproducts, AG1 produced a small but significant increase in ammonium production ($p = 0.02$; 5.1% increase) but did not promote significant branched-chain SCFA production. These data suggest fermentation occurred in a transplanted human colonic microbiota and these processes were enhanced by the AG1 nutritional supplement. Ultimately, AG1 showed preclinical evidence as a synbiotic given the significant increases in total SCFA production, acetate, propionate, and other metabolic byproducts of fermentation.

Keywords: synbiotic; gut microbiome; fermentation; SCFA; foundational nutrition; probiotics; phytonutrients; prebiotics; supplementation

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1. Introduction

Prebiotics were first defined in 1995 as non-digestible food components that beneficially impact the human host by supporting the growth and function of bacteria in the colon, resulting in beneficial health effects when consumed in a sufficient amount [1]. Ultimately, what drive the health benefits of prebiotics are the metabolites produced. Among these metabolites, short-chain fatty acids (SCFAs) are the highest produced and best understood [2]. SCFAs are largely associated with a plethora of localized health benefits in the intestines such as improving the gut barrier integrity, glucose and lipid metabolism, colonization resistance against enteric pathogens, and the regulation of the gut-associated lymphoid tissue [3–5]. SCFAs also confer health benefits systematically including anti-inflammation, immunoregulation, anti-obesity, anti-diabetes, anti-cancer, cardioprotective,

hepatoprotective, and neuroprotective activities [6,7]. Traditionally, fructo-oligosaccharides (FOSs) and galactooligosaccharides (GOSs) have been the two major groups of prebiotics and have been the most extensively studied [2]. However, there is growing evidence that some phytonutrients (e.g., phenolic acids, flavonoids, etc.) can act as prebiotics [8–11]. Because of this, not all prebiotics are exclusively fibers and not all dietary fibers exhibit prebiotic effects [12].

Probiotics are live microorganisms that, when consumed, provide health benefits to the host [13]. Some of the best-characterized and most well understood species of probiotics come from the genera *Lactobacillus* and *Bifidobacterium* [14]. Synbiotics are the combination of prebiotics with probiotics with the intent to promote synergism between the two products, amplifying a beneficial effect [15,16]. It is generally accepted that the intent of the prebiotics in synbiotics is to support the health benefit of the probiotic; however, prebiotics can also yield health benefits through the metabolism of the resident colonic microbiota [17].

Recently, there has been increased public interest in taking prebiotics, probiotics, and synbiotics as part of a health and wellness regimen, in tandem with multivitamins or other supplements. In 2021 alone, it is estimated that 1 in 20 American adults were using non-food-based prebiotics, probiotics, and synbiotics [18]. Due to the growing popularity and use of these products, there is also a growing concern about the misuse of these terms [15]. In 2020, The International Scientific Association of Probiotics and Prebiotics (ISAPP) published a position stand where they defined synbiotics as "a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host" [16]. Therefore, there is a growing need to perform efficacy testing in conjunction with typical industry standard testing on over-the-counter prebiotics, probiotics, and synbiotics, to determine if a synbiotic demonstrates the potential to confer benefits to a host.

AG1® (AG1) is a novel foundational nutrition supplement that is designed to exert a synbiotic effect. AG1 contains vitamins and minerals, where micronutrients have been known to influence gut microbiome composition [19,20]. As previously mentioned, AG1 contains various phytonutrients which could act as prebiotics [8–11] as well as inulin, a more recognized prebiotic fiber. AG1 contains adaptogens, like ashwagandha, and functional mushrooms (e.g., shiitake and reishi mushrooms) which may influence gut microbiome metabolic function [21,22]. When taken together, there may be a potential prebiotic effect which synergistically supports the two probiotics found in AG1: *Lactobacillus acidophilus* UALa-01 and *Bifidobacterium bifidum* UABb-10 [23].

The primary objective of this investigation is to determine whether AG1's formulation supports a prebiotic effect. We anticipate that in vitro experimentation using the Simulator of Human Intestinal Microbial Ecosystem (SHIME®) model will demonstrate physical and metabolic evidence of enhanced fermentation occurring in ex vivo human gut microbiota. Briefly, complex carbohydrate prebiotics undergo fermentation and typically yield SCFAs and gases [24]. Therefore, we identify various aspects of a successful prebiotic as (1) having the ability to undergo fermentation; (2) fermentation produces beneficial metabolites; (3) does not yield evidence of a perceived harmful effect (unfavorable metabolites like branched SCFAs (bSCFAs). In the current experiment, these were evaluated as the production of gases and changes in pH (physical evidence of fermentation), the production of SCFAs, and the production of ammonium and bSCFAs.

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. A recommended dose of AG1 designed for human consumption is 12 g per serving. For the current experiment, a dose of 6 g/reactor was chosen to mitigate physical complications that would impact the mechanical and biological factors

of the SHIME® model. The placebo group only received the blank control medium used to deliver AG1. The ingredients in AG1 are available online [25] and in a supplemental figure here (Figure S1) and have undergone evaluation and verification via NSF testing (Ann Arbor, MI, USA) to ensure the product meets strict quality, purity, safety, and label accuracy standards [26].

2.2. Test Gastrointestinal Tract System

We employed the SHIME® model adapted from Molly et al., 1993 with a focus on the stomach, small intestine, and proximal colon [27]. The experimental design for SHIME is well documented in other publications [28]. For a detailed schematic overview, Duysburgh et al., 2022 provided a figure in their publication on the SHIME model [29]. Briefly, the model simulated the human gastrointestinal tract using two bioreactors. 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 a reactor over time followed by standardized bile acid and enzyme solutions to simulate the human small intestine. Prespecified pH and incubation times have been established to mimic in vivo conditions for each compartment of the human gastrointestinal tract mentioned [30].

2.3. Gastric Phase

The sample was incubated at 37 °C for 45 min with constant mixing via stirring and maintained at a pH of 2.0. Pepsin was added with the activity being standardized by measuring the absorbance increase at 280 nm of TCA-soluble components produced upon digestion of hemoglobin. Pepsin and phosphatidylcholine levels 4-fold lower than the fed condition (1000 U/mL and 0.02 mM, respectively) were added [31]. The background medium used contained only salts and mucins recommended by the consensus method, with NaCl and KCl reaching concentrations of ~50 mM and ~7 mM, respectively.

2.4. Small Intestine Phase

The contents from the gastric phase were mixed via stirring, and the pH was automatically increased from 2.0 to 6.5 in the duodenal phase. Mixing occurred for 27 min at a constant pH of 6.5. The jejunal and ileal phase was 3 h in total with a constant pH of 7.0 at 37 °C. At the end of the duodenal phase, there was a simulated absorptive process using a dialysis approach. The dialysis approach 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 were a raw animal pancreatic extract (pancreatin) containing all the relevant enzymes in a specific ratio, upon measuring trypsin activity (TAME-assay) to normalize for specific activity. The activity was set at 1.12 TAME U/mL (5-fold lower compared to the fed conditions) [31]. 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, which were 5-fold lower compared to the fed conditions [31]. The bile salts used during the small intestine phase were derived from bovine bile which is a closer match to human than porcine in terms of tauro- and glycocholate. Based on Riethorst et al., 2016, the bile salt concentration was decreased by a factor of 3 with a general amount of 3.33 mM bovine bile extract being supplemented [31]. Following the 3 h small intestine phase, the luminal content was collected, and this undigested fraction was used to initiate the short-term colonic batch simulations.

2.5. Short-Term Colonic Batch Simulations

A batch fermentation design was utilized for the current experiment to allow for donor-specific reactions and to quantify the total metabolic output from microbial fermentation without fear of metabolite loss in a dynamic fermentation design. The short-term colonic

incubations were conducted using 53 mL of colonic medium, 7 mL of fecal inoculum, and 10 mL of the luminal content from the small intestine phase. The 53 mL of colonic medium contained host- and diet-derived substances including, amongst others, peptone, yeast extract, and L-cysteine. This colonic medium was mixed with 10 mL of the luminal content as metabolic input for the microbial fermentation. A representative amount (7 mL) of fresh fecal matter from three seemingly healthy adults (BMI = 18.5–24.9; no antibiotic use in previous 4 months; not diagnosed with a specific disease known to be associated with altered gut microbiome states) was used as a fecal inoculum. The choice to use fresh fecal inocula over established in vitro microbiota was deliberate to ensure all gut microbiota species from the gut microbiome were present and none were outcompeted or washed out due to an in vitro environment. This also allowed the fermentation potential and direct effect on the microbiome community of the test product to be measured. The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University Hospital Ghent (reference number ONZ-2022-0267). Inocula from each donor were used as independent samples (n = 3) with three biological replicates per donor to account for biological variation. The fresh inocula were manipulated on the day of the experiment according to ProDigest's in-house protocols. The bioreactors were made anaerobic by flushing with nitrogen gas and were incubated for 48 h at 37 °C under shaking conditions (90 rpm). After starting incubation, the pH and gas was determined at 0, 1, 24, and 48 h. Gas pressure was measured using a hand-held pressure indicator (WIKA, CPH6200, Lawrenceville, GA USA) with a transmitter (WIKA, CPT6200, Lawrenceville, GA USA). Ammonium was determined colorimetrically (AQ300 Discrete Analyzer) using the indophenol blue spectrophotometric method at 0, 24, and 48 h. At 0 h, only the blank control samples were analyzed. The quantitative analysis of the various SCFAs (including the bSCFAs) was performed using a Shimadzu GC2030 gas chromatograph with an autosampler and flame-ionization detector. The GC injector port was installed with an enduro blue injector septum and inlet liner with a standard split. The used inlet liner was specified as follows: A Shimadzu 221-75189, glass insert liner with quartz wool and deactivation. A BP21 (FFAP) GC column was used with a length of 30 m, inside diameter of 0.32 mm, and film thickness of 0.25 µm. Nitrogen gas was used as the carrier gas with a flow rate of 1.82 mL/min, and at the inlet, the sample was split 10:1. The injection volume was set at 1 µL. The run time was programmed at 11.33 min. The oven temperature was programmed as follows: initial temperature 110 °C; temperature ramp 6 °C/min to 160 °C; held for 3 min. The injector and detector temperatures were both set at 200 °C. The peak area output signal was computed via integration using Lab solutions DB software. The isolation of short-chain fatty acids (including isobutyrate, isovalerate, and isocaproate together as total bSCFAs) was performed using liquid–liquid extraction [32]. These were assessed at 0, 1, 24, and 48 h. At 0 h, only the blank control samples were analyzed.

2.6. 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) Averages were obtained from the three biological replicates per donor for each of the variables collected. 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. Paired t-tests were conducted for the net change from baseline to 1 h (except for ammonia), baseline to 1 day (24 h), and overall (baseline to 48 h). Observed p-values were reported, with p-values less than 0.05 used to indicate significance.

3. Results

3.1. Physical Evidence of Microbial Fermentation

To evaluate whether fermentation was able to occur, physical evidence of microbial fermentation was examined (Figure 1). To investigate the net change in acid and base formation, changes in local pH were measured (Figure 1A). The results showed that the

net change in local pH did not significantly differ between conditions during the first hour (p = 0.09), first day (p = 0.20), or overall during colonic simulation (p = 0.38). To investigate the net change in gas production, local gas pressure was measured (Figure 1B). There was statistical evidence that suggested AG1 increased the amount of gas produced during colonic simulation compared to the control. Gas production significantly increased during the first hour (p = 0.002), the first day (p = 0.005), and over the total 48 h of colonic simulation (p = 0.002) compared to the control.

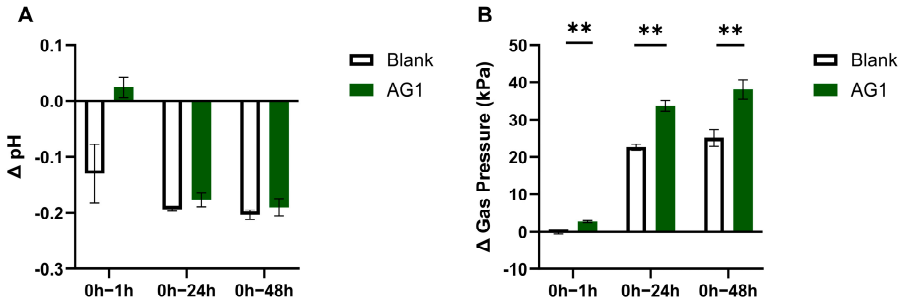

Figure 1. AG1 significantly changed the physical environment due to increased fermentation compared to the blank control. Panel (**A**) shows the change in local pH and panel (**B**) shows that net change in gas pressure. Statistical analysis included paired *t*-tests. Data are shown as mean and standard error of the mean. ** $p < 0.01$.

3.2. Byproducts from Protein Fermentation

Two major metabolic byproducts of protein fermentation include ammonia production and the formation of bSCFAs (Figure 3). Ammonia production was not statistically different between conditions (Figure 3A) during the first day (p = 0.13) but was significantly elevated overall in AG1 compared to the control (p = 0.02). Changes in the bSCFAs (Figure 3B) were used to measure the fermentation of branched amino acids. There were no differences in bSCFAs during the first hour (p = 0.44), the first day (p = 0.72), or overall during colonic simulation (p = 0.23) between conditions.

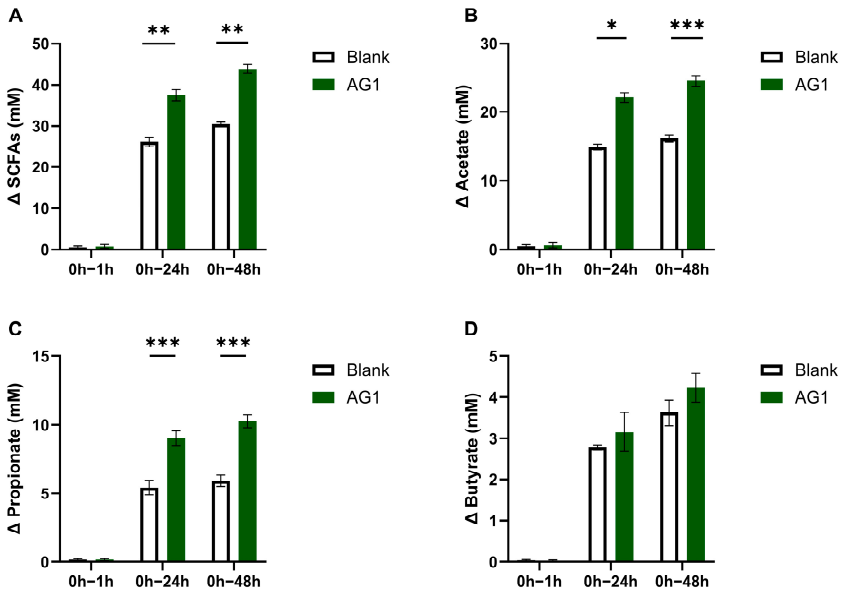

Figure 2. AG1 produced significantly greater increases in typical byproducts of carbohydrate fermentation compared to the blank control. Panel (**A**) shows the net change in total SCFAs, and panels (**B**–**D**) show the net change in acetate, propionate, and butyrate respectively. Statistical analysis included paired *t*-tests. Data are shown as mean and standard error of the mean. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.3. Byproducts of Fermentation

The major and most beneficial byproducts of microbial fermentation include SCFAs, and SCFA production was measured (Figure 2). Generally, there were no significant increases in SCFA production during the first hour of colonic simulation. However, the total SCFA production as well as the production of acetate and propionate were significantly increased during the first day ($p = 0.002$, $p = 0.01$, $p = 0.0001$, respectively) and over 48 h of colonic simulation ($p = 0.002$, $p = 0.001$, $p = 0.0001$, respectively). There were no significant differences in butyrate production during the first hour ($p = 0.18$), the first day ($p = 0.48$), or overall during colonic simulation ($p = 0.45$) compared to the control condition.

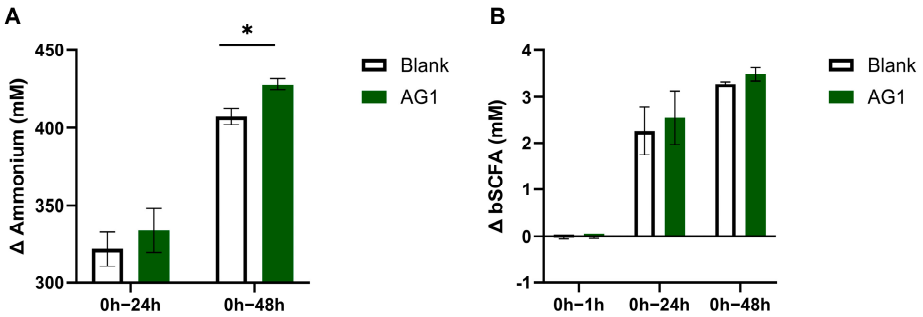

Figure 3. AG1 modestly increases some, but not all, typical byproducts of protein fermentation compared to the blank control. Panel (**A**) shows the net change in ammonium and panel (**B**) shows the net change in total bSCFAs. Statistical analysis included paired *t*-tests. Data are shown as mean and standard error of the mean. * $p < 0.05$.

4. Discussion

The overarching goal of the current study was to evaluate the capacity of AG1 to be fermentable and exert a prebiotic effect. Prebiotics must be able to undergo fermentation; the prebiotic(s) in AG1 should be fermented by the resident microbiota and yield typical byproducts of fermentation [33]. Gas is a typical byproduct of fermentation, and thus, changes in gas formation can indirectly measure the extent of fermentation. Changes in pH as a result of fermentation are largely driven by the formation of lactic acid and SCFAs as they are acids [34]. There was not enough evidence in this model to suggest that AG1 supplementation caused a significant change in the local pH relative to the blank control's effect on the local pH. It is important to note that both the AG1 treatment and the blank control did cause an overall decrease in the pH. Regardless, AG1 did significantly increase the amount of gas being formed throughout the colonic simulation, suggesting that an increase in fermentation was occurring. The lack of significant change in local pH when compared to the control treatment could have been caused by various factors. Primarily, byproducts of fermentation are acidic and basic in nature. Therefore, the net production of both basic and acidic byproducts could neutralize any change in pH. Moreover, many byproducts of microbial metabolism act as buffers. This is so that the overall metabolic output of the microbiome does not create unfavorable conditions [35]. Therefore, it is possible that the lack of significant change in pH was largely impacted by metabolites that may or may not necessarily have been measured in the current experimental design.

The second aspect which defines a successful prebiotic and probiotic effect is the production of beneficial metabolites resulting from fermentation. SCFAs are one of the major byproducts of microbial fermentation and are the major metabolites that are formed in the colon [36]. Acetate (2 carbon), propionate (3 carbon), and butyrate (4 carbon) are the major chemicals that comprise the SCFAs with known health benefits. This experiment demonstrated that AG1 treatment could increase the overall production of SCFAs. As anticipated, there was not an immediate increase in the total concentration of SCFAs, but they did significantly increase after the first day. This is largely because lactic acid is produced immediately and acts as an intermediate before being subsequently fermented further into SCFAs. This primarily is achieved by microbes from the phylum Firmicutes [37].

Regardless, total SCFA production was significantly increased in AG1 compared to control. This is worthwhile to note as they can exert many benefits to the host [3–7]. AG1 treatment significantly increased the overall production of acetate and propionate but not butyrate. Consistent with what was observed for the total SCFAs, it appeared that the majority of the SCFAs were produced during the first day of the simulation. Failure to find significant butyrate production despite an overall significant increase in total SCFA production was not surprising considering that of all the SCFAs produced in humans, approximately 60% is acetate, 25% is propionate, and 15% is butyrate [38].

The third aspect of a successful prebiotic or probiotic is that there should not be evidence that consuming the product would exert significant adverse effects on the host. A common side effect of many prebiotics and some probiotics is bloating, gastrointestinal distress, and other related symptoms which are generally attributed to gas production. While gas production is a necessary byproduct of fermentation, too much and too rapid gas production can be an overall negative attribute. It is important to note that we did observe a significant increase in gas production during the first hour. Pham and colleagues also used the SHIME® model to assess ways to treat a fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (FODMAP)-rich diet to reduce the overall gastrointestinal distress derived from the FODMAPs. They observed that the FODMAP diet alone changed the amount of gas produced by roughly ~25 kPa relative to the blank control and that enzymatically treating the FODMAPs reduced the gas production to similar to the blank control [39]. AG1 caused gas production of only ~10% of that caused by FODMAP-rich diets and was comparable to that of an enzymatically treated FODMAP diet. Therefore, we believe there is not sufficient evidence to suggest AG1 consumption would lead to significant bloating, gas, or gastrointestinal distress related to gas production.

We found that AG1 supplementation caused a modest but significant increase in ammonium production overall during the colonic simulation. There was no effect observed for bSCFAs. Despite the negative association of protein fermentation to negative health conditions, there is some nuance to these biochemical processes that must be considered. Contrary to carbohydrates, protein fermentation is a bit more biochemically complex, with the byproducts being extremely dependent on the monomer as well as the mechanism of the biochemical reaction. Microbes can either perform metabolic deamination reactions on proteins/amino acids to produce a carboxylic acid (SCFAs/bSCFAs) and ammonia or they can undergo a decarboxylation reaction to produce an alkyl amine and carbon dioxide [40]. Regarding the monomer, when branched amino acids (valine, leucine, and isoleucine) are the substrate, a bSCFA is produced in conjunction with ammonia, whereas the non-branched amino acids will yield SCFAs and ammonia [41]. Note that SCFAs are understood to be the most abundant byproduct of amino acid fermentation in the gut microbiome [42]. Therefore, there is more ammonia production than alkyl amine production, and alkyl amines are more toxic than ammonia to humans. Ammonia production is also beneficial to the microbiome to some extent, as ammonia production largely dictates the pH of the colonic luminal environment and acts as a buffer against the acidification caused by SCFAs [35]. Microbes can also rapidly assimilate ammonia for amino acid biosynthesis [43]. Together, there are some clear benefits of ammonia in the gut microbiome ecosystem, but the full extent of how protein fermentation impacts human health remains to be fully elucidated [44]. Humans evolved systems to quickly remove excess microbially derived ammonia and process it into urea for excretion. So, while there was a significant increase in ammonia production, it is not possible to evaluate whether it is excessive or detrimental in a human due to the lack of physiological mechanisms to regulate ammonia production in the intestinal lumen. What effect this has on humans must be further explored in a clinical setting, but we do not anticipate a detrimental effect based on the current data.

The utilization of the SHIME® model has some strengths that make it an excellent first model for use in this experiment. Because this experiment was set to investigate a potential prebiotic effect of AG1, some variables were necessary to be observed in vitro. For example, the production of gas would be difficult to quantify in a clinical setting and

would rely on subjective questionnaire data. These data can be problematic as humans have varying thresholds for symptom complaints. Clinical data can also be confounded by a lack of protocol adherence, missed clinical visits, and overall intrasubject variability that would require larger sample sizes that were beyond a proof-of-concept experimental design. Despite these strengths, there are also some limitations in the current study. One limitation is the acute, short-term nature of the dosing paradigm used in this experimental period. AG1 is a daily nutritional supplement and is meant to be taken daily. Therefore, there is an anticipated change in many of these variables when observed in a chronic experimental paradigm. If there are changes in the microbial community, then it is anticipated that the formation of the SCFAs as well as other byproducts of fermentation would change. This also highlights the need for a metagenomic investigation of the microbial community. Additionally, the current study does not measure the acute effect of AG1 supplementation on alterations in community composition and function. Future studies would benefit from the inclusion of metagenomic data. Another limitation is the small sample size of the study given the fact that the human microbiome is incredibly diverse and metabolically complex, leading to large amounts of variability. Finally, it is also important to recognize that in vitro work does not replace the need for clinical experimentation and seldom translates perfectly to what is expected in a human study. However, the SHIME® model does an impressive job trying to emulate the physiological and biological environment of the digestive tract, but it cannot replace the value of clinical testing. Despite this, the data collected are invaluable and help establish the proof-of-concept that AG1 can undergo fermentation as a prebiotic and can lead to significant increases in beneficial SCFAs like acetate and propionate. To fully investigate the effect of these increased metabolites on humans, work needs to be carried out in clinical studies.

5. Conclusions

From the data, it is suggested that AG1 exerts a prebiotic effect expected from the combination of fiber and phytonutrients, but also from other aspects of the product like the micronutrients. This was made evident from the formation of gas (physical evidence of fermentation), the production of beneficial metabolites (total SCFAs, acetate, propionate), and the lack of a significant detrimental effect (modest gas production within the first hour, slight ammonia production, and non-significant bSCFA production). All of these factors are derived from the microbiota's ability to ferment the components of AG1. Despite these conclusions, further investigations must be carried out in a clinical setting to confirm AG1's synbiotic effects. Future work should also include investigations into what effect AG1 has on the community structure and function of the gut microbiome. Metagenomic investigations would help us understand the prebiotic effect of AG1 by measuring changes in microbial metabolism. Finally, despite some of the limitations to this study, the current study suggests AG1 can be fermentable and should undergo subsequent clinical studies for further investigation on the potential health benefits.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nutraceuticals3040035/s1, Figure S1: AG1® Product Guide.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University Hospital Ghent (reference number ONZ-2022-0267).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Upon reasonable request, data from the corresponding author are made available; however, certain data may not be made available owing to privacy issues.

Conflicts of Interest: J.R.T. and P.A.S. have conducted sponsored research on nutritional supplements. J.R.T., T.O.K., P.A.S., T.M.M. and R.E. are employees of Athletic Greens (AG). M.G., M.M. and C.D. are employees of ProDigest BVBA. The authors declare that this study received funding from AG. The funders were involved in the design of the study, in the writing of the manuscript, and in the decision to publish the results.

References

1. Gibson, Y.; Roberfroid, M.B. Dietary Modulation of the Human Colonie Microbiota: Introducing the Concept of Prebiotics. *J. Nutr.* **1995**, *125*, 1401–1412. [CrossRef] [PubMed]
2. Bamigbade, G.B.; Subhash, A.J.; Kamal-Eldin, A.; Nyström, L.; Ayyash, M. An Updated Review on Prebiotics: Insights on Potentials of Food Seeds Waste as Source of Potential Prebiotics. *Molecules* **2022**, *27*, 5947. [CrossRef]
3. Nogal, A.; Valdes, A.M.; Menni, C. The Role of Short-Chain Fatty Acids in the Interplay between Gut Microbiota and Diet in Cardio-Metabolic Health. *Gut Microbes* **2021**, *13*, 1897212. [CrossRef]
4. Chambers, E.S.; Preston, T.; Frost, G.; Morrison, D.J. Role of Gut Microbiota-Generated Short-Chain Fatty Acids in Metabolic and Cardiovascular Health. *Curr. Nutr. Rep.* **2018**, *7*, 198–206. [CrossRef]
5. Blaak, E.E.; Canfora, E.E.; Theis, S.; Frost, G.; Groen, A.K.; Mithieux, G.; Nauta, A.; Scott, K.; Stahl, B.; van Harsselaar, J.; et al. Short Chain Fatty Acids in Human Gut and Metabolic Health. *Benef. Microbes* **2020**, *11*, 411–455. [CrossRef]
6. Xiong, R.G.; Zhou, D.D.; Wu, S.X.; Huang, S.Y.; Saimaiti, A.; Yang, Z.J.; Shang, A.; Zhao, C.N.; Gan, R.Y.; Li, H. Bin Health Benefits and Side Effects of Short-Chain Fatty Acids. *Foods* **2022**, *11*, 2863. [CrossRef]
7. Ríos-Covián, D.; Ruas-Madiedo, P.; Margolles, A.; Gueimonde, M.; De los Reyes-Gavilán, C.G.; Salazar, N. Intestinal Short Chain Fatty Acids and Their Link with Diet and Human Health. *Front. Microbiol.* **2016**, *7*, 185. [CrossRef]
8. Carrera-Quintanar, L.; Roa, R.I.L.; Quintero-Fabián, S.; Sánchez-Sánchez, M.A.; Vizmanos, B.; Ortuño-Sahagún, D. Phytochemicals That Influence Gut Microbiota as Prophylactics and for the Treatment of Obesity and Inflammatory Diseases. *Mediat. Inflamm.* **2018**, *2018*, 9734845. [CrossRef]
9. Kan, J.; Wu, F.; Wang, F.; Zheng, J.; Cheng, J.; Li, Y.; Yang, Y.; Du, J. Phytonutrients: Sources, Bioavailability, Interaction with Gut Microbiota, and Their Impacts on Human Health. *Front. Nutr.* **2022**, *9*, 960309. [CrossRef]
10. Dingeo, G.; Brito, A.; Samouda, H.; Iddir, M.; La Frano, M.R.; Bohn, T. Phytochemicals as Modifiers of Gut Microbial Communities. *Food Funct.* **2020**, *11*, 8444–8471. [CrossRef]
11. Peterson, C.T.; Sharma, V.; Uchitel, S.; Denniston, K.; Chopra, D.; Mills, P.J.; Peterson, S.N. Prebiotic Potential of Herbal Medicines Used in Digestive Health and Disease. *J. Altern. Complement. Med.* **2018**, *24*, 656–665. [CrossRef]
12. Kaur, A.P.; Bhardwaj, S.; Dhanjal, D.S.; Nepovimova, E.; Cruz-martins, N.; Kuča, K.; Chopra, C.; Singh, R.; Kumar, H.; Şen, F.; et al. Plant Prebiotics and Their Role in the Amelioration of Diseases. *Biomolecules* **2021**, *11*, 440. [CrossRef]
13. Kechagia, M.; Basoulis, D.; Konstantopoulou, S.; Dimitriadi, D.; Gyftopoulou, K.; Skarmoutsou, N.; Fakiri, E.M. Health Benefits of Probiotics: A Review. *ISRN Nutr.* **2013**, *2013*, 481651. [CrossRef]
14. Salminen, S.J.; Gueimonde, M.; Isolauri, E. Symposium: Innate Immunity and Human Milk Probiotics That Modify Disease Risk 1. *J. Nutr.* **2005**, *135*, 1294–1298. [CrossRef]
15. Pandey, K.R.; Naik, S.R.; Vakil, B.V. Probiotics, Prebiotics and Synbiotics—A Review. *J. Food Sci. Technol.* **2015**, *52*, 7577–7587. [CrossRef]
16. Salminen, S.; Collado, M.C.; Endo, A.; Hill, C.; Lebeer, S.; Quigley, E.M.M.; Sanders, M.E.; Shamir, R.; Swann, J.R.; Szajewska, H.; et al. The International Scientific Association of Probiotics and Prebiotics (ISAPP) Consensus Statement on the Definition and Scope of Postbiotics. *Nat. Rev. Gastroenterol. Hepatol.* **2021**, *18*, 649–667. [CrossRef]
17. Roy, S.; Dhaneshwar, S. Role of Prebiotics, Probiotics, and Synbiotics in Management of Inflammatory Bowel Disease: Current Perspectives. *World J. Gastroenterol.* **2023**, *29*, 2078–2100. [CrossRef]
18. O'Connor, L.E.; Gahche, J.J.; Herrick, K.A.; Davis, C.D.; Potischman, N.; Vargas, A.J. Nonfood Prebiotic, Probiotic, and Synbiotic Use Has Increased in US Adults and Children From 1999 to 2018. *Gastroenterology* **2021**, *161*, 476–486.e3. [CrossRef]
19. Barone, M.; D'Amico, F.; Brigidi, P.; Turroni, S. Gut Microbiome–Micronutrient Interaction: The Key to Controlling the Bioavailability of Minerals and Vitamins? *BioFactors* **2022**, *48*, 307–314. [CrossRef]
20. Hadadi, N.; Berweiler, V.; Wang, H.; Trajkovski, M. Intestinal Microbiota as a Route for Micronutrient Bioavailability. *Curr. Opin. Endocr. Metab. Res.* **2021**, *20*, 100285. [CrossRef]
21. Dey, A.; Chatterjee, S.; Kumar, V. Triethylene Glycol-like Effects of *Ashwagandha* (*Withania Somnifera* (L.) Dunal) Root Extract Devoid of Withanolides in Stressed Mice. *AYU (Int. Q. J. Res. Ayurveda)* **2018**, *39*, 230. [CrossRef]
22. Zhao, J.; Hu, Y.; Qian, C.; Hussain, M.; Liu, S.; Zhang, A.; He, R.; Sun, P. The Interaction between Mushroom Polysaccharides and Gut Microbiota and Their Effect on Human Health: A Review. *Biology* **2023**, *12*, 122. [CrossRef]

23. Raman, M.; Ambalam, P.; Kondepudi, K.K.; Pithva, S.; Kothari, C.; Patel, A.T.; Purama, R.K.; Dave, J.M.; Vyas, B.R.M. Potential of Probiotics, Prebiotics and Synbiotics for Management of Colorectal Cancer. *Gut Microbes* **2013**, *4*, 181–192. [CrossRef]
24. Holscher, H.D. Dietary Fiber and Prebiotics and the Gastrointestinal Microbiota. *Gut Microbes* **2017**, *8*, 172–184. [CrossRef]
25. Athletic Greens International Ingredients. Available online: https://drinkag1.com/en (accessed on 18 August 2023).
26. Travis, J.; Lattimore, L.G.; Harvey, M.; Frey, T. NSF International's Role in the Dietary Supplements and Nutraceuticals Industries. In *Nutraceutical and Functional Food Regulations in the United States and around the World*; Academic Press: Cambridge, MA, USA, 2019; pp. 147–158. [CrossRef]
27. Molly, K.; Vande Woestyne, M.; Verstraete, W. Applied AFwrobiology Biotechnology Development of a S-Step Multi-Chamber Reactor as a Simulation of the Human Intestinal Microbial Ecosystem. *Appl. Microbiol. Biotechnol.* **1993**, *39*, 254–258. [CrossRef]
28. Bell, L.; Whyte, A.; Duysburgh, C.; Marzorati, M.; Van den Abbeele, P.; Le Cozannet, R.; Fança-Berthon, P.; Fromentin, E.; Williams, C. A Randomized, Placebo-Controlled Trial Investigating the Acute and Chronic Benefits of American Ginseng (Cereboost®) on Mood and Cognition in Healthy Young Adults, Including in Vitro Investigation of Gut Microbiota Changes as a Possible Mechanism of Action. *Eur. J. Nutr.* **2022**, *61*, 413–428. [CrossRef]
29. Duysburgh, C.; Verstrepen, L.; Broeck, M.V.D.; Righetto, Z.; Perez, M. Investigation of Enterogermina's Protective and Restorative Mechanisms on the Gut Microbiota with PPI, Using SHIME Technology. *Nutrients* **2023**, *15*, 653. [CrossRef]
30. Mackie, A.; Rigby, N. InfoGest Consensus Method. In *The Impact of Food Bioactives on Health: In Vitro and Ex Vivo Models*; Springer International Publishing: New York, NY, USA, 2015; pp. 13–22, ISBN 9783319161044.
31. Riethorst, D.; Mols, R.; Duchateau, G.; Tack, J.; Brouwers, J.; Augustijns, P. Characterization of Human Duodenal Fluids in Fasted and Fed State Conditions. *J. Pharm. Sci.* **2016**, *105*, 673–681. [CrossRef]
32. De Boever, P.; Deplancke, B.; Verstraete, W. Nutritional Methodology Fermentation by Gut Microbiota Cultured in a Simulator of the Human Intestinal Microbial Ecosystem Is Improved by Supplementing a Soygerm Powder 1. *J. Nutr.* **2000**, *130*, 2599–2606. [CrossRef]
33. Dahiya, D.; Nigam, P.S. Probiotics, Prebiotics, Synbiotics, and Fermented Foods as Potential Biotics in Nutrition Improving Health via Microbiome-Gut-Brain Axis. *Fermentation* **2022**, *8*, 303. [CrossRef]
34. Den Besten, G.; Van Eunen, K.; Groen, A.K.; Venema, K.; Reijngoud, D.J.; Bakker, B.M. The Role of Short-Chain Fatty Acids in the Interplay between Diet, Gut Microbiota, and Host Energy Metabolism. *J. Lipid Res.* **2013**, *54*, 2325–2340. [CrossRef] [PubMed]
35. Shen, T.C.D.; Albenberg, L.; Bittinger, K.; Chehoud, C.; Chen, Y.Y.; Judge, C.A.; Chau, L.; Ni, J.; Sheng, M.; Lin, A.; et al. Engineering the Gut Microbiota to Treat Hyperammonemia. *J. Clin. Investig.* **2015**, *125*, 2841–2850. [CrossRef] [PubMed]
36. Silva, Y.P.; Bernardi, A.; Frozza, R.L. The Role of Short-Chain Fatty Acids from Gut Microbiota in Gut-Brain Communication. *Front. Endocrinol.* **2020**, *11*, 25. [CrossRef] [PubMed]
37. Duncan, S.H.; Louis, P.; Flint, H.J. Lactate-Utilizing Bacteria, Isolated from Human Feces, That Produce Butyrate as a Major Fermentation Product. *Appl. Environ. Microbiol.* **2004**, *70*, 5810–5817. [CrossRef]
38. Liu, H.; Wang, J.; He, T.; Becker, S.; Zhang, G.; Li, D.; Ma, X. Butyrate: A Double-Edged Sword for Health? *Adv. Nutr.* **2018**, *9*, 21–29. [CrossRef] [PubMed]
39. Pham, V.T.; Steinert, R.E.; Duysburgh, C.; Ghyselinck, J.; Marzorati, M.; Dekker, P.J.T. In Vitro Effect of Enzymes and Human Milk Oligosaccharides on FODMAP Digestion and Fecal Microbiota Composition. *Nutrients* **2023**, *15*, 1637. [CrossRef]
40. Fan, P.; Li, L.; Rezaei, A.; Eslamfam, S.; Che, D.; Ma, X. Metabolites of Dietary Protein and Peptides by Intestinal Microbes and Their Impacts on Gut. *Curr. Protein Pept. Sci.* **2015**, *16*, 646–654. [CrossRef]
41. Heimann, E.; Nyman, M.; Pålbrink, A.K.; Lindkvist-Petersson, K.; Degerman, E. Branched Short-Chain Fatty Acids Modulate Glucose and Lipid Metabolism in Primary Adipocytes. *Adipocyte* **2016**, *5*, 359–368. [CrossRef]
42. Oliphant, K.; Allen-Vercoe, E. Macronutrient Metabolism by the Human Gut Microbiome: Major Fermentation by-Products and Their Impact on Host Health. *Microbiome* **2019**, *7*, 91. [CrossRef]
43. Portune, K.J.; Beaumont, M.; Davila, A.M.; Tomé, D.; Blachier, F.; Sanz, Y. Gut Microbiota Role in Dietary Protein Metabolism and Health-Related Outcomes: The Two Sides of the Coin. *Trends Food Sci. Technol.* **2016**, *57*, 213–232. [CrossRef]
44. Wu, S.; Bhat, Z.F.; Gounder, R.S.; Ahmed, I.A.M.; Al-Juhaimi, F.Y.; Ding, Y.; Bekhit, A.E.D.A. Effect of Dietary Protein and Processing on Gut Microbiota—A Systematic Review. *Nutrients* **2022**, *14*, 453. [CrossRef] [PubMed]