Journal of Functional Foods 119 (2024) 106319

Contents lists available at ScienceDirect

Journal of Functional Foods

journal homepage: www.elsevier.com/locate/jff

Changes in the fecal polar metabolome due to AG1 supplementation in the SHIME® model: A proof of principle study

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ARTICLE INFO

Keywords:
Gut microbiome
Metabolome
Nutritional supplements
Gut-brain axis

ABSTRACT

Metabolomics is a critical approach to fully understand the metabolic repercussions of a nutritional supplement on the gut microbiota. Here, a proof of principle study was conducted via a metabolomic survey of polar metabolites to explore the effect of AG1 on the metabolic output of the human gut microbiota, *in vitro*, using the Simulator of the Human Intestinal Microbial Ecosystem® (SHIME). Aliquots were isolated from the fecal slurry at baseline (0 h), 1 h, 24 h, and 48 h of colonic fermentation simulation. Chemical analysis at these timepoints detected a total of 165 metabolites with 57, 21, and 14 specific metabolites being significantly altered by AG1 relative to the blank controls at 1 h, 24 h, and 48 h, respectively. Many of these metabolites likely resulted from AG1 directly as a supplement, but several of these metabolites also likely arose specifically from microbial metabolism.

1. Introduction

In recent decades, there has been a substantial increase in research focused on the gut microbiome. Furthermore, the emergence of the "omics" era (e.g., metagenomics, transcriptomics, proteomics, etc.) in life sciences has been instrumental in the characterization of systems biology and biological processes. Currently, metagenomics is the most widely implemented human gut microbiome studies providing valuable insights into the composition, diversity, and functional potential of gut microbiome. However, there is a level of nuance which accompanies the interpretation of these "omic" data. That is, changes in the metagenome, transcriptome, or even proteome do not directly characterize the dynamic and detailed metabolic change in living organisms (Qiu et al., 2023). Rather, they give strong and detailed information on the mechanisms of change that result in altered metabolic profiles. Metabolomics is part of the "omics" era of the life sciences which attempts to characterize and quantify small molecules in a given sample (Idle & Gonzalez, 2007). This is particularly important when it comes to diet and dietary interventions (Ryan et al., 2013; Koulman and Volmer, 2008). Consequently, Precision Nutrition is a developing field within nutritional science that seeks to leverage a combination of multiple omics technologies, such as metagenomics and metabolomics, as a comprehensive approach to understand how individuals react to specific dietary interventions (Naureen et al., 2022). This approach can be particularly helpful to assess both the host response as well as the host's microbiomes response to nutritional interventions. Moreover, with a general observation in dietary inadequacies, there is a growing need for tools to adequately understand the impact of diet, dietary short comings, and human health.

Diet is closely related to health and disease with epidemiological studies demonstrating that high-quality diets are associated with longevity and disease incidence (Shan et al., 2020; Gao et al., 2021; Goshen et al., 2022). However, the diet of United States adults, in relation to the US Dietary Guidelines for Americans, is characterized by excess intakes of refined grains, sodium, and added sugar and sub-optimal intakes of fruits, vegetables, and whole grains (Shams-White et al., 2023). These poor-quality diets likely explain the sub-optimal intakes in several micronutrients (e.g., Vitamin E, calcium, and magnesium) consumed by adults 19 years of age and older (USDA, Agricultural Research Service, 2023). Many Americans are aware of their

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https://doi.org/10.1016/j.jff.2024.106319
Received 26 March 2024; Received in revised form 14 June 2024; Accepted 16 June 2024
Available online 27 June 2024
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poor diet quality and choose to consume dietary supplements in an attempt to maintain or improve their health and close their nutrient gaps (Bailey, Gahche, Miller, Thomas, & Dwyer, 2013). Hence, a large percentage (>57 %) of US adults reported taking a dietary supplement in the last 30-days based on cross-sectional data from 2017 to 2018 (Mishra, Stierman, Gahche, & Potischman, 2021). While multivitamins are the most common dietary supplement consumed (Mishra et al., 2021), products aimed at improving gut health have gained in popularity in recent years given the increased awareness regarding the impact of gut health on systemic well-being (Nguyen et al., 2020). Subsequently, there has been an increase in studies assessing the effects of dietary supplements, pre-, probiotics, and diet (e.g., micronutrients) on the gut microbiome due to their potential and known effects on the microbiome (Gibson and Roberfroid, 1995; Turroni et al., 2018; Yang et al., 2020).

A majority of the research has focused on how supplements, particularly single ingredient supplements containing micronutrients or probiotics, impact the diversity and structure of the gut (Yang et al., 2020; Kristensen et al., 2016; Mörschbächer et al., 2023). These metagenomic outcomes provide important data demonstrating how dietary supplements may alter the alpha and beta-diversity of the gut microbiome. However, few studies have employed metabolomics to assess how dietary supplements may alter the metabolites produced by the gut microbiota. Metabolomic data is needed to understand what metabolites are being increased or decreased following supplementation that could have downstream effects on host health. Moreover, there is a paucity of research assessing multi-ingredient supplements when they are the most commonly consumed dietary supplements by US adults. Advances in technology and methodologies allow for *in vitro* assessment of dietary supplements on the human gut microbiome using a gastrointestinal (GI) simulation model [Simulator of the Human Intestinal Microbial Ecosystem® (SHIME)] that closely mimics the human tract in a tightly controlled environment (Van den Abbeele et al., 2010; Vanden Bussche et al., 2015; Molly et al., 1993).

Due to the common use of multi-ingredient supplements by US adults and lack of metabolomic data on dietary supplements we evaluated the metabolic byproducts of a novel nutritional supplement. More specifically, we used a multi-ingredient supplement, AG1, containing vitamins, minerals, adaptogens, prebiotics, and probiotics in an *in vitro* model. Our aim was to assess the metabolites produced using the SHIME *in vitro* model of the human GI tract using global metabolomics as part of a proof of principle study. Due to the complexity of AG1, the inclusion of 75 ingredients, and lack of other studies on a similar product being available, we opted for proof of principle study to determine the effect on the fecal metabolome in a highly controlled model prior to a human clinical. We hypothesized and demonstrated that a novel synbiotic containing many active ingredients would have significant impacts on the gut microbiome and alter the metabolites produced and aimed to test this through a metabolomic analysis of the polar metabolies.

2. Materials and methods

2.1. Experimental design and model

AG1® (AG1; AG1, 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. A dose of 6 g/reactor was chosen for the current experiment 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 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 (Travis, Lattimore, Harvey, & Frey, 2019).

Briefly, we employed the SHIME model (van de Wiele, van den Abbeele, Ossieur, Possemiers, & Marzorati, 2015). This model was chosen as it emulates the chemical and physiological conditions of the human gastrointestinal tract to simulate realistic conditions anticipated. Moreover, this model allows for consistent digestive conditions and minimizes interpersonal variation that could confound the potential results. AG1 was exposed to a gastric phase in which the test product was subjected to normal stomach physiological conditions. Following the gastric phase, physiological conditions were shifted towards conditions of the duodenum briefly and then transferred to a dialysis membrane to emulate absorption of the digested fraction. The non-digested fraction was subsequently transferred to a mixture of colonic medium and human fecal inocula.

A total of three stool donors were included in the study. Each donor was determined to be healthy, with a BMI between 18.5–24.9. Donors were screened for any diseases which could result in a dysbiotic community structure (e.g., irritable bowel disease, Parkinson's disease, diabetes, etc.) and did nor take antibiotics within 4 months before stool donation. The donors consisted of 1 male and 2 females aged 26 years, 28 years, and 34 years respectively. Stool donations were freshly collected in containers containing an AnaeroGen (Thermo Fisher Scientific, Waltham, MA USA) sachet to maintain anaerobic atmosphere. A fecal inoculum was prepared by making a 1:13 (w/v) mixture of the fecal sample with anaerobic phosphate buffer (K2HPO4 8.8 g/l; KH2PO4 6.8 g/l; sodium thioglycolate 0.1 g/l; sodium dithionite 0.015 g/l). After homogenization (10 min, BagMixer® 400, Interscience, Louvain-La-Neuve, Belgium) and removal of big particles via centrifugation (2 min, 500*g*), an inoculum corresponding to 10 % (v/v) was added to the different incubations.

Colonic simulations were performed under physiological conditions of the proximal colon for 48 h. 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). Written informed consent was obtained from all stool donors.

2.2. Metabolite extraction, separation, and quantification

Changes in the metabolome brought on upon by microbial fermentation was observed using previously validated methodology (Vanden Bussche et al., 2015; De Paepe et al., 2018). This was done using a global metabolic profiling specific for 409 polar metabolites using UHPLC-HRMS. Aliquots for the metabolic profiling were isolated after 0 (baseline), 1, 24, and 48 h of colonic simulation. At 0 h, only the blank control samples were analyzed. Each aliquot underwent a liquid extraction protocol. Aliquots consisted of 300 µL sample was extracted per bioreactor by the addition of ultrapure water. After shaking the resulting mixture and performing subsequent centrifugation, the supernatant was collected and filtered through a polyvinylidene fluoride filter (0.22 µm pore size). From the purified extract, a 10-µL aliquot was injected into the UHPLC-HRMS system. Prior to analysis, the MS system was calibrated according to the manufacturer's guidelines (Thermo Fisher Scientific) to warrant accurate mass measurements (<5 ppm mass deviation) in both positive and negative ionization mode.

Chromatographic separation was achieved on a Vanquish quaternary pumping system (Thermo Fisher Scientific, USA), equipped with an Acquity HSS T3 C18 column (1.8 µm, 150 × 2.1 mm) (Waters Corporation, UK). A binary solvent system consisting of ultrapure water and acetonitrile, both acidified with 0.1 % formic acid, was used at a constant flow rate and by applying a gradient profile. Detection was performed on a Q-Exactive™ standalone bench top quadrupole-Orbitrap high-resolution mass spectrometer (Thermo Fisher Scientific, USA), which was preceded by heated electrospray ionization (HESI-II source) in polarity switching mode. The instrument was operated at a resolution of 140,000 full width at half maximum and in full-scan mode (m/z scan range of 53.4–800), meaning that all ions detected were registered and no fragmentation was implemented. To ensure quality control for the chromatographic and mass spectrometry performance, internal standards were evaluated in tandem with the target metabolites.

Xcalibur software version 4.0.27.21 (Thermo Fisher Scientific, USA) was used to identify and subsequently quantify the captured metabolites. A metabolite was considered below the limit of detection (LOD) or limit of quantification (LOQ) if the metabolite's observed peak area was below 250,000 arbitrary units. Information on the general noise level, stability of retention time, and the coefficient of variance as determined for the repeated measure of iQC-samples were additional features to assess the reliability of detection and quantification. Following peak integration, the area ratio was determined for each metabolite by calculating the ratio between the area of the metabolite and that of the most suited internal standard. A sample-specific normalization that was based on internal standards as well as an intra-batch normalization strategy was applied based on the iQC samples.

For normalization, the analytical strategy included five deuterated internal standards, which were eluted at various times during the 18-min sample run and thus were representative for the entire retention time range of the LC-MS methodology. The area ratios were calculated by dividing the area of a metabolite within a sample by the area of the most suited internal standard in that same sample. Selection of the most suited internal standard for each metabolite was based on the similarity in retention time as well as the capability of that internal standard to correct for metabolite-specific instrumental variation. The latter was defined based on the set of iQC-samples. Having this normalization strategy of internal standards available corrects for variation during extraction and instrumental analysis, and allows for reliable comparisons between biological replicates, donors, and treatment groups. Metabolite specific variations at the instrumental level were further addressed by usage of the iQC-samples.

2.3. Metabolite analysis and statistical approaches

For univariate statistical analysis, all values that were considered below the LOQ were replaced with half of the lowest detected relative abundance that was above the LOQ. This approach may improve the data analysis by providing a more accurate representation of the data without the need to consider some metabolites as missing data. Normality was assessed using the Shapiro-Wilk normality test and based on a brief review of the quantile–quantile plots. Homogeneity of variances was assessed using Levene's test. Outliers were determined using Z-scores with a cut off for values greater than 3.5 or less than −3.5. Comparisons between the AG1 treated samples and the blank controls were assessed using paired Student's *t*-test. Statistical significance was determined to be achieved when *p*-values were less than 0.05. For discussion purposes, trends were determined to be observed when *p*-values were between 0.0501 and 0.1000. All statistical analyses were conducted using GraphPad Prism version 10.1.1.

3. Results

A total of 165 metabolites were identified in the fecal inocula (Supplemental Table 1, Supplemental Fig. 1). Of them, only 57 metabolites showed statistical difference between the AG1 treated samples and the blank control samples for at least one time point (Table 1). The 1 h time point appeared to be the most significantly altered with 37 metabolites yielding statistically significant results. A total of 21 and 14 metabolites were observed to be statistically significant between the two groups at 24 h and 48 h respectively.

Table 1
List of metabolites that were significantly different between AG1 and blank control.

Metabolite	1 h	24 h	48 h
1-Palmitoyl-*glycero*-3-phosphoethanolamine	**0.0369***	0.1983	0.3850
2,6-Diaminopimelic acid	**0.0424**	**0.0094**	0.0504
2-Piperidinone	0.6762	**0.0130**	0.0744
3,4-Dihydroxyphenylalanine	**0.0153**	0.2676	0.8888
5-Hydroxytryptophan	**0.0263**	0.4226	0.4226
6-Phosphogluconic acid	**0.0234**	0.1998	0.4226
7-Ketodeoxycholic acid	**0.0052**	0.4103	0.4226
7-Methylguanine	**0.0002**	0.2954	0.2142
Acetic acid	0.3486	**0.0068**	**0.0220**
Adenine	0.7723	**0.0428**	0.0635
Anandamide	**0.0127**	**0.0165**	0.2270
Aspartic acid	0.4376	**0.0446**	**0.0031**
Cadavarine	0.6521	0.1301	**0.0437**
Chenodeoxycholic acid (CDCA)	**0.0192**	0.0732	0.1006
Cholic acid (CA)	**0.0013**	0.3430	0.7679
Choline	**0.0280**	0.0756	0.0825
Citric acid	**0.0000**	**0.0284**	0.1585
Citrulline	0.6769	**0.0078**	0.1127
Daidzein	**0.0074**	0.6213	0.8189
Deoxycholic acid (DCA)	**0.0192**	0.0732	0.1006
Formononetin	**0.0177**	0.4226	0.4226
Fructose/Galactose	**0.0144**	0.0970	**0.0420**
Genistein	**0.0000**	0.4226	0.4226
Glucose/Mannose	**0.0026**	1.0000	1.0000
Glycerol	**0.0212**	0.1544	0.4226
Guanine	0.5753	0.3237	**0.0403***
Hexadecamide/Palmitamide	**0.0346**	0.9013	0.5763
Hypoxanthine	0.7684	**0.0192**	0.5352
Indole-3-propionic acid	0.4226	**0.0381**	0.1378
Kynurenine	0.3011	**0.0020**	0.0573
Leucylglycine	0.2377	**0.0244**	**0.0374**
Lysine	0.4521	**0.0027**	**0.0066**
Malic acid	**0.0241**	0.2906	0.2225
Methionine	0.4390	**0.0122**	**0.0096**
Methionine sulfoxide	**0.0136**	0.0686	**0.0364**
N6-acetyllysine	**0.0127**	**0.0049**	**0.0293**
N-acetylgalactosamine/N-acetylglucosamine	0.2028	**0.0274**	0.1481
Nicotinic acid/Picolinic acid	**0.0066**	0.2918	0.0770
Oleamide	**0.0264**	0.6031	0.0880
Oxoglutaric acid/Ketoglutaric acid	**0.0000**	1.0000	1.0000
Pantothenic acid	**0.0040**	0.3565	0.3682
Proline	0.5499	0.1121	**0.0103**
Pyruvic acid	**0.0228**	0.1962	0.6808
Saccharic acid	**0.0135**	1.0000	1.0000
Serotonin (5-hydroxytryptamine)	**0.0197**	0.2961	0.3115
Spermidine	**0.0194**	0.0520	0.5585
Suberic acid	**0.0148**	**0.0142**	0.1103
Sucrose/Lactose/Maltose	**0.0325**	0.4226	0.4226
Theobromine	**0.0166**	**0.0013**	**0.0066**
Threonine	0.7041	**0.0016**	0.1699
Trehalose	**0.0408**	0.4226	0.4226
Trimethylamine	0.3888	0.0772	**0.0458**
Tyramine	0.3535	0.0971	**0.0104**
Tyrosine	**0.0006**	0.4372	0.4945
Urocanic acid	0.2287	**0.0288**	0.1519
Ursodeoxycholic acid (UDCA)	0.4299	**0.0055**	0.1565
Xanthosine	**0.0431**	1.0000	1.0000

P-values determined by paired Student's *t*-test at each respective time point. Bolded values represent statistically significant values ($P < 0.05$).
*Denotes time points with statistically significant metabolite amounts which are greater for the blank control group relative to the AG1 treated group.

4. Discussion

The aim of this study was to investigate changes in the gut metabolome in response to the administration of a novel nutritional supplement, AG1, utilizing an *in vitro* model of the human digestive tract as part of a proof of principle study. Here, a total of 165 polar metabolites were identified in the blank control and AG1 treated fecal inocula with 57 metabolites yielding statistically significant differences between the two treatment groups. Of these metabolites, the significant differences were observed in the AG1 treatment at the 1 h (37 metabolites), 24 h (21 metabolites) and 48 h time points (14 metabolites). Only 2 of the statistically significant metabolites (1-palmitoyl-*glycero*-3-phosphoethanolamine and guanine) were elevated in the blank control treated group when compared to AG1 treated group.

Results of the study indicated that 55 metabolites were elevated for the AG1 treated samples. AG1 contains whole food ingredients as well as herbal extracts. Therefore, elevations in simple carbohydrates (e.g.,

fructose/galactose, glucose/mannose, and sucrose/lactose/maltose), organic compounds (e.g., malic acid and saccharic acid), as well as some amino acids (e.g., proline, citrulline, and aspartic acid), especially at the 1 h time point, was anticipated. Identification of specific phytochemicals was also anticipated. Primary examples include formononetin, an isoflavone from Astragalus (Tian, Wang, & Tian, 2022), genistein and daidzein, isoflavones found in soy (Soukup, Engelbert, Watzl, Bub, & Kulling, 2023), as well as theobromine and xanthosine, xanthine derivatives commonly found in cocoa (Martínez-Pinilla, Oñatibia-Astibia, & Franco, 2015). With respect to these phytochemicals, the elevations of these metabolites could translate into health benefits to the host associated with anti-inflammatory and antioxidant properties (Tian et al., 2022; Soukup et al., 2023; Martínez-Pinilla et al., 2015), but would need to be demonstrated in a clinical setting. Lastly, it is also important to note that some vitamins (e.g., nicotinic acid/picolinic acid and pantothenic acid) were also observed to be significantly increased in the AG1 treated samples consistent with the MVM aspect to the product.

Although it's evident that a considerable number of the identified metabolites originated from AG1 as a nutrient source, it's noteworthy that microbial metabolism played a role in influencing the levels of these metabolites. This is likely demonstrated with several amino acid metabolites detected in our analysis. There were several metabolites which were not significantly observed until the 24 h or the 48 h timepoint. Further, we observed chemically modified amino acid species or byproducts of peptide degradation, like N6-acetyllysine and leucylglycine. Beyond amino acid metabolism, there are other metabolic pathways in which the gut microbiota plays a critical role in shaping.

The gut microbiome plays a significant role in bile acid metabolism, making it a crucial metabolic pathway and source of subsequent metabolites (Collins, Stine, Bisanz, Okafor, & Patterson, 2023) due to the impact it has on shaping the gut microbial community (Ridlon, Kang, Hylemon, & Bajaj, 2014). Here, we demonstrated that AG1 has an impact on the microbiota which resulted in differential metabolism of bile acid species. Cholic acid was noted to be significantly elevated at the 1 h period while chenodeoxycholic acid was significantly elevated at the 1 h period with a trended increase at the 24 h period as well. Deoxycholic acid demonstrated a similar pattern with significance at the 1 h period and only a trended increase at the 24 h period while ursodeoxycholic acid only was significantly elevated at the 24 h period. Also of note, only cholic acid diminished over time while chenodeoxycholic acid, deoxycholic acid, and ursodeoxycholic acid increased over time. The role each of these bile acids plays in human biology is quite complex. Deoxycholic acid can exert immunomodulatory effects by acting upon G protein-coupled bile acid receptor 1 (GPBAR1) on intestinal and liver macrophages thus suppressing inflammatory processes (Lou et al., 2014; Biagioli et al., 2017). Beyond this, GPBAR1 has been shown to influence the cardiovascular system, the hypothalamic–pituitary–adrenal axis, and even the central nervous system (Shulpekova et al., 2022).

The gut-brain axis is a bidirectional communication network which involves complex interactions between the gut microbiota in various cognitive domains (e.g., executive function, mood). We observed additional metabolites which have been shown to modulate the host's nervous system function through this gut-brain axis (Carabotti et al., 2015; Appleton, 2018). Namely, the metabolism of tryptophan into the serotonin and kynurenine metabolic pathways as well as the dopamine pathway was altered by the administration of AG1 to the gut microbiota. Regarding the dopamine pathway, there was a significant increase in tyrosine at 1 h and a significant increase of 3,4-dihydroxyphenylalanine (L-dopa) at 1 h. However, no effect was observed for dopamine despite an elevated mean relative abundance of dopamine at both the 24 h and 48 h time points. This suggested that AG1 provided metabolic substrates which were necessary to produce L-dopa and likely dopamine, but further experimentation with larger sample sizes is needed.

Tryptophan can be metabolized into various neurologically relevant metabolites by the gut microbiota. In the serotonin metabolic pathway, 5-hydroxytrptophan and serotonin were significantly elevated the 1 h. Serotonin was shown to have an elevated mean relative abundance for the 24 h and 48 h time points but was not statistically significant. For the kynurenine pathway, kynurenine was significantly elevated at 24 h and a trend was observed at 48 h. In the indole pathway, indole-3-propionic acid was shown to be significantly elevated at 24 h with an increased mean relative abundance at 48 h but failed to reach statistical significance. Serotonin is perhaps the most recognizable of these metabolic pathways. The indole pathway can be considered beneficial to the host as indole-3-propionic acid has been shown to attenuate microglial inflammation and likely promoted neuronal function in an elderly human population (Kim, Jung, Hwang, & Shin, 2023). Kynurenine is known to modulate brain development (Goeden et al., 2017) and cognition (Pocivavsek, Elmer, & Schwarcz, 2019) by acting as an endogenous antagonist to the N-methyl-D-aspartate receptor. Whether AG1 supplementation would modulate these neurologically relevant metabolites in humans, whether they escape the colonic microenvironment, and whether they can impact human mood and cognition still needs to be investigated in a clinical study.

Regarding metabolites which were elevated in the blank control, the observation that at the 1 h timepoint that 1-palmitoyl-*glycero*-3-phosphoethanolamine was lower for AG1 vs. blank control was striking. This phospholipid is commonly found in wine grapes (*Vitis vinifera)* (Center, 2024) and AG1 contains grape seed extract which is made from wine grapes (Gupta et al., 2020). Upon further examination, the mean relative amount of 1-palmitoyl-*glycero*-3-phosphoethanolamine is higher for the AG1 treated samples but was not considered to be statistically significant. The other metabolite, guanine, did not demonstrate a similar pattern. Rather, at all the time points the relative amount of guanine was higher for blank control treated groups but only demonstrated statistical significance at the 48 h. The significance of this observation needs further experimentation to fully elucidate the findings and we cannot provide a plausible hypothesis for why this phenomenon occurred.

It should be noted that relative abundances and not absolute concentrations were determined for the various metabolites. As the baseline metabolic profiles may be different for the various donors included in this study, a paired experimental design and subsequent paired statistical analysis were essential. Paired statistics may address the issue of different baseline metabolic profiles between donors, thereby focusing on the overall net treatment effects. Furthermore, it may also address the potential differences in the contribution of the fecal metabolome (as part of the inoculum) at the start of the *in vitro* simulation while the fecal microbiome adjusted to the experimental conditions. By using the same donors for both the treatment and placebo groups, differences in baseline fecal metabolomes were also controlled for. This paired statistical approach may increase the statistical power and circumvent some experimental limitations, such as the relatively low number of biological replicates and potential differences in fecal metabolite due to inter-donor differences as opposed to a treatment effect.

This current study evaluated the metabolic outcomes of AG1 when subjected to human microbiota *in vitro*. Since many variables can confound the metabolic output of the microbiota (e.g., diet, exercise, lifestyle factors, etc.) we chose a controlled experimental design to understand the effect of AG1 on the gut microbiota's metabolome. Due to this design, however, there are several factors which may limit the extent to which our findings can be generalized to a larger population. Namely, this was a proof of principle study that looked at the effect of AG1 in three independent stool samples in biological triplicates. Because of this, in conjunction with the knowledge that the human gut microbiome is extremely varied, the generalizability of these data is limited and subsequent studies *in vitro* with larger sample sizes as well as the clinical evaluation is needed to confirm these findings. Inclusion of more participants also could allow stratification based on sex (as disclosed by the stool donors), which could elucidate sex specific differences brought on to the microbiome due to the ingestion of AG1. Future clinical investigations would benefit from the addition of serum metabolomic

analyses in larger cohorts to account for variation in the metabolic potential of the microbiome.

5. Conclusions

In an *in vitro* model of the human digestive tract, we were able to show that the non-absorbed fraction of AG1 acts as a source of nutrients for the gut microbiota via a proof of principle study. The majority of significant changes on the metabolome occurred during the first hour of colonic simulation and many metabolites noted were consistent with known ingredient sources in AG1 (e.g., carbohydrates and phytochemicals). However, we observed more nuanced metabolite results at later phases of the colonic simulation likely stemming from microbial metabolic pathways, many of which could be attributed to the gut-brain axis. Many of these metabolic alterations could be associated with beneficial effects on the host's health. This experiment was designed in such a way to screen for key biomarkers of interest within the fecal metabolome. However, follow up clinical studies are needed to validate these preliminary and preclinical findings. Subsequent experiments can employ targeted and absolute quantification for key metabolites (e.g., those related to tryptophan metabolism) noted to be impacted by AG1. Moreover, targeted experimentation could elucidate the biological mechanisms, changes in pathways, and interconnectivity between the altered metabolites. However, due to the limited sample sizes in the current study, future studies with larger sample sizes powered adequately based off values obtained from this preliminary study are warranted to reliably evaluate the effect of AG1 on the gut metabolome as well as evaluate AG1′s efficacy in humans who exhibit substantial variation in responses to nutritional supplements.

6. 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).

Funding

This research was funded by AG1 (AG1, Carson City, NV).

Informed consent statement

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

CRediT authorship contribution statement

Trevor O. Kirby: Writing – review & editing, Writing – original draft, Conceptualization. **Philip A. Sapp:** Writing – review & editing, Writing – original draft, Conceptualization. **Jeremy R. Townsend:** Writing – review & editing, Writing – original draft, Conceptualization. **Marlies Govaert:** Writing – review & editing, Investigation. **Cindy Duysburgh:** Investigation. **Massimo Marzorati:** Investigation. **Tess M. Marshall:** Writing – review & editing, Conceptualization. **Ralph Esposito:** Writing – review & editing, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: 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 AG1. M.G., M.M. and C.D. are employees of ProDigest BVBA. The authors declare that this study received funding from AG1. The funders were involved in the design of the study, in the writing of the manuscript, and in the decision to publish the results.

Data availability

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2024.106319.

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