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Fermented brown rice beverage distinctively modulates the gut microbiota in Okinawans with metabolic syndrome: A randomized controlled trial

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ABSTRACT

Accumulating evidence to date suggests that brown rice is superior to white rice in regard to its beneficial impact on a number of risk factors of the metabolic syndrome (MetS). However, little is known about the influence of fermented brown rice beverage on the gut microbiota in humans. We therefore hypothesized that its impact would beneficially alter the gut microbiota composition of patients with MetS. Using a 4-week randomized, single-arm study design, subjects (n = 40) were advised to consume a daily fermented brown rice beverage (BA) or fermented white rice beverage (WA) as a replacement of their main meal. Clinical and anthropometric measurements as well as fecal samples were collected at baseline and immediately after completion of the intervention. Gut microbiota was analyzed using 16S ribosomal RNA sequencing and capillary electrophoresis-time-of-flight mass spectrometry was used to measure plasma short-chain fatty acids. Interestingly, ingestion of BA in contrast to WA resulted in a unique elevation in the abundance of number of beneficial species belonging to the Clostridia class, associated with reduced inflammation, and increased short-chain

Abbreviations: BA, fermented brown rice beverage; CE-TOFMS, capillary electrophoresis-time-of-flight mass spectrometry; HbA1c, hemoglobin A1c; HDL-C, high-density lipoprotein cholesterol; HMT, Human Metabolome Technologies; LDA, linear discriminant analysis; LDL-C, low-density lipoprotein cholesterol; LEfSe, linear discriminant analysis effect size; MetS, metabolic syndrome; rRNA, ribosomal RNA; SEM, standard error of the mean; TG, triglycerides; T2DM, type 2 diabetes mellitus; SCFAs, short-chain fatty acids; WA, fermented white rice beverage.

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fatty acid production: *Lactobacillales bacterium DJF B280* ($P = .005$), *Butyrate producing bacterium A2 207* ($P = .012$), and *Firmicutes bacterium DJF VP44* ($P = .038$). This study demonstrates that consumption of BA is effective to beneficially modulate the gut microbiota compared with WA in patients with MetS.

1. Introduction

Consumption of whole grains, which contain the bran and germ and are rich in fiber, micronutrients, and phytochemicals, are inversely associated with conditions of the metabolic syndrome (MetS) [1], a clustering of cardiometabolic risks that increase the overall likelihood of chronic disease development [2]. Brown rice, a whole grain high in fiber and phytosterols such as γ-oryzanol and once widely consumed by Asian population groups such as the Japanese, has demonstrated blood glucose and lipid-lowering effects as well as anti-inflammatory and antiobesity activities in both humans and animals [3]. Data obtained from Health Professionals Follow-up Study and the Nurses' Health Study I and II revealed that a higher intake of brown rice was associated with a lower risk of type 2 diabetes mellitus (T2DM), whereas white rice was associated with an increased risk [4]. Interestingly, the same researchers reported that by substituting 50 g/d of white rice for brown rice, T2DM risk was lowered by 16% [5]. In Japanese subjects, twice-daily brown rice consumption has been shown to improve glycemic control as indicated by significant reductions in postprandial blood glucose, hemoglobin A1c (HbA1c), and glycoalbumin levels [6]. Our team also found that daily ingestion of brown rice for 8 weeks in subjects with MetS in Okinawa, the Southern tip of Japan, was able to significantly lower postprandial blood glucose levels and protect endothelial function [7]. Regarding some of the possible mechanisms at play, research in our laboratory has shown that the bioactive component, specifically and abundantly contained in brown rice, γ-oryzanol, can reduce endoplasmic reticulum stress in the hypothalamus [8], protect pancreatic β cells against apoptosis [9], and act as an inhibitor of DNA methyltransferases in the brain reward system in mice [10].

Diet and its impact on the gut microbiota have been recognized as significant contributors in the pathogenesis of MetS and associated chronic diseases [11]. Diets rich in whole grains have been shown to exert positive effects on the gut microbiota, most notably by increasing microbial diversity and promoting growth of microbial species that can ferment fiber, producing short-chain fatty acids (SCFAs) [12]; which play diverse roles in appetite and body weight regulation as well as reducing inflammation and aiding gut barrier maintenance [13, 14]. Even short-term consumption of whole grains as part of a daily diet results in observable positive effects on the gut microbiota, SCFAs, and a number of markers of immune and gut-mediated inflammation [15, 16]. Notably, habitual brown rice consumption in Japanese adults led an increased abundance of *Faecalibacterium prausnitzii*, a well-known producer of the SCFA butyrate and member of the Clostridia class, a group of bacteria known for their therapeutic and anti-inflammatory effects [17, 18].

In Japan, much of the good health and longevity experienced is thought to be related to the traditional Japanese diet (Washoku) [19], which is low in calories and nutrient dense, with meals centered around rice as the staple carbohydrate, offering a diverse range of fermented foods such as miso, which are fermented with the fungus *Aspergillus oryzae* (koji) [20]. Notably, amazake, a traditional Japanese fermented rice beverage made using rice koji, contains B vitamins and number of bioactive substances, with some research suggesting favorable effects on metabolic parameters [21, 22]. As a result of rapid Westernization and complex historical factors, Okinawa currently has one of the highest rates of obesity and T2DM in Japan, along with a concomitant decrease in consumption of brown rice and Amazake [23]. However, little is known about the impact of traditional foods such as brown rice or amazake on the gut microbiota in relation to metabolic health, highlighting the need for studies that contribute to a better understanding of the relationship between diet and the gut microbiota, and therapeutic application of food in disease. The aim of this study was therefore to explore the impact of the fermented brown rice beverage (amazake) on the gut microbiota and major microbial metabolites in Okinawan subjects with MetS. We hypothesized that acute consumption of brown rice amazake, in contrast to white rice amazake, would result in an increased abundance of beneficial bacteria and potentially, plasma SCFA levels, supporting gut health and likely improving aspects of MetS.

2. Materials and Methods

2.1. *Subjects*

This study targeted outpatients with the MetS that visited the Okinawa Health Promotion Foundation from 1 June 2014 to 31 May 2015. Inclusion criteria were as follows; body mass index $\geqq$ 25 and any 2 of the following cardiometabolic risk factors: (1) hypertriglyceridemia (serum triglyceride [TG] concentration $\geqq$ 150 mg/dL; (2) low serum high-density lipoprotein cholesterol (HDL-C) (HDL-C concentration <40 mg/dL; (3) elevated blood pressure (systolic blood pressure $\geqq$130 mm Hg and/or diastolic blood pressure $\geqq$85 mm Hg); or (4) high fasting glucose (serum glucose concentration $\geqq$100 mg/dL). Exclusion criteria were as follows: (1) subjects taking medications that may influence study results (including antidiabetic medications known to impact gut microbiota such as metformin); (2) infectious disease or history of recent operation or injury;

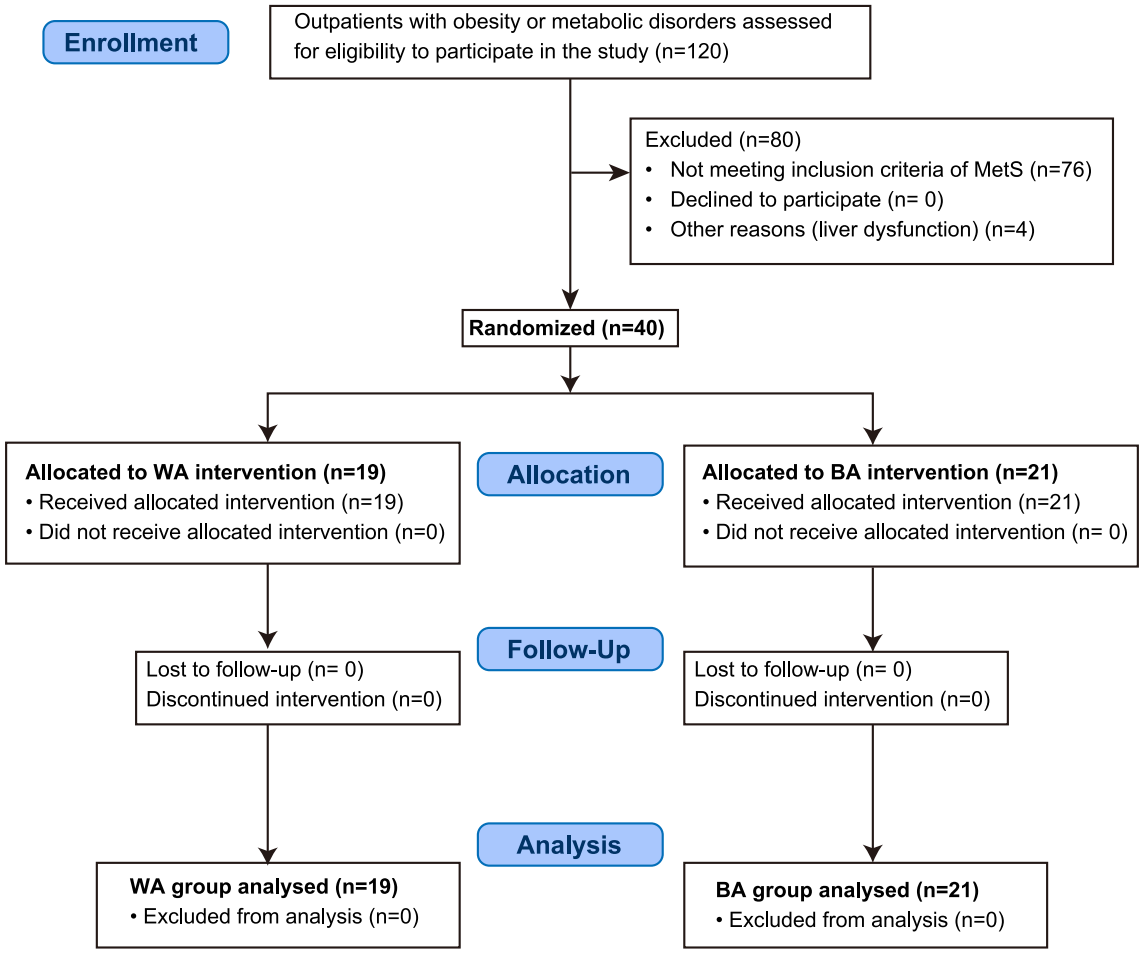

Fig. 1 – Flow diagram depicting the study design.

(3) pregnant or nursing women; and (4) severe renal or liver dysfunction.

The present study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human participants were approved by the Ethics Committee for the "Okinawa Health Promotion Foundation" (approved at 20140529) and registered at the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR registration no. UMIN000017485). Written informed consent was obtained from all participants. All subjects were obliged to report any serious or unexpected adverse events as well as declare any relations to those included in the intervention immediately to either the principal investigator and/or research ethics committee.

2.2. *Study design*

This study was a single arm, 4-week randomized intervention trial with a total of 40 participants recruited for the study (Fig. 1). Of the 120 outpatients assessed for the study, 4 were excluded because of moderate or severe liver dysfunction and 76 did not meet the criteria for the MetS. Once selected, subjects were randomly allocated to 1 of 2 groups; receiving a 350-g pack of either white rice amazake (WA) (n = 19; 11 males and 8 females), or brown rice amazake (BA) (n = 21; 9 males and 12 females), and advised to consume this beverage in replace of their main meal, once per day for 4 weeks, with the amount of test beverage based on findings from our previous work [7]. Anthropometric and clinical examinations were conducted at baseline (week 0), as well as blood samples and fecal (stool) samples collected at baseline (week 0) and at the end of the intervention period (week 4) to assess the impact on gut microbiota composition (primary outcome). A total of 3 plasma samples (for SCFA analysis) and 2 fecal samples from the WA group, as well as 2 plasma samples and 4 fecal samples from the BA group, were unable to be collected/processed.

2.3. *Formulation of WA and BA*

White rice and brown rice amazake were developed and commercialized by the Aizu Tempo Co. (Fukushima, Japan) for use in the present study. Using either white rice (for formulation of WA) or brown rice (for formulation of BA), rice was first cracked on its surface physically and then washed with water at 10 to 15°C and immersed for 12 to 18 hours. After immersion, the rice was then steamed at 0.36 to 0.48 MPa (90-110°C), for 50 to 90 minutes. Fermentation was then carried out by an automatic apparatus (stainless steel, made by YAEGAKI Food & System, Inc., Japan). The apparatus was subsequently filled with steamed rice and *Aspergillus oryzae* and kept at 33 to 35°C

Table 1 – Nutrient composition of experimental beverages

Nutrient	WA	BA
Energy, kcal	315	340
Protein, g	4.9	6
Fat, g	0.7	3.2
Carbohydrate, g	72.1	71.8
Total fiber, g	1.1	3.2
γ-oryzanol, mg	1.8	32

Per 350-g pack.
BA, brown rice amazake; WA, white rice amazake.

for 41 to 46 hours. A total of 70 to 160 L of water was then put into the mixture apparatus (stainless steel, made by YAESU Co., Ltd., Japan), and heated to 75 to 85°C with 30 to 60 kg steamed rice. At 50 to 70°C, 40 to 80 kg of rice koji was then added into the mixture apparatus, mixed, and equalized. The mixture was then maintained at 50 to 70°C for 6 to 12 hours to enable the rice starch granules to be converted to glucose. Finally, the undiluted, fermented white or brown rice beverages (WA or BA) were prepared and packaged, ready for consumer use. Table 1 shows the nutritional composition of the test drinks.

2.4. *Measurement of biochemical parameters*

Fasting blood samples were collected from participants at baseline (week 0) and at the end of the intervention (last day of week 4). Samples were collected in either heparin (for subsequent SCFA analysis) or ethylene-diamine-tetra-acetic acid-containing tubes and then centrifuged at 3300 rpm for 10 minutes to obtain plasma and serum and sent to SRL Laboratories for analyses. Using an automatic analyzer JCA-BM8000 series (Japan Electronics Co., Japan), 0.5 mL of serum was analyzed to measure total cholesterol, low-density lipoprotein cholesterol (LDL-C), HDL-C, and TG, and using an automatic analyzer JCA-BM9000 series (Japan Electronics Co., Japan), 0.5 mL of sodium fluoride plasma was analyzed to measure blood glucose levels. Insulin was measured by analyzing 0.4 mL of serum using the fully automated chemiluminescent enzyme immunoassay system Lumipulse series (Fujirebio Co., Ltd.) HbA1c was measured by analyzing 2.0 mL of blood (with sodium fluoride) using the HLC-723G8.

2.5. *Analyses of plasma SCFAs*

The SCFAs were measured using capillary electrophoresis-time-of-flight mass spectrometry (CE-TOFMS). Metabolites were first extracted by adding 50 µL of heparinized plasma to 450 µL methanol solution and stirred for about 10 seconds. To this, 500 µL of chloroform and 200 µL of Milli-Q water were added, stirred, and the centrifuged (2300×*g*, 4°C, 5 minutes). After centrifugation, the aqueous layer was transferred to one 400-µL ultrafiltration tube (Ultra-Free MC PLHCC, Human Metabolome Technologies [HMT], Japan) and then centrifuged (9100×*g*, 4°C, 120 minutes) and subjected to ultrafiltration. The filtrate was dried and solidified using a decompression dryer and then dissolved in 50 µL of Milli-Q water. SCFA measurements were the carried out through a facility service at HMT. Briefly, CE-TOFMS analysis was carried out using an Agilent CE capillary electrophoresis system equipped with an Agilent 6210 time-of-flight mass spectrometer (Agilent Technologies, Inc., Santa Clara, CA, USA). The systems were controlled by Agilent G2201AA ChemStation software version B.03.01 for CE (Agilent Technologies) and MassHunter for LC (Agilent Technologies). The spectrometer was scanned from *m/z* 50 to 1000 and peaks were extracted using MasterHands, an automatic integration software (Keio University, Tsuruoka, Yamagata, Japan), to obtain peak information including *m/z*, peak area, and migration time for CE-TOFMS [24]. Signal peaks corresponding to isotopomers, adduct ions, and other product ions of known metabolites were excluded, and the remaining peaks were annotated according to HMT's metabolite database based on their *m/z* values and migration times. Areas of the annotated peaks were then normalized to internal standards (L-Methionine Sulfone for cationic metabolites and D-Camphor-10-sulfonic for anionic metabolites) and sample amount to obtain relative levels of each of the SCFAs. Measurement was performed assuming 100% uniformity with recovery efficiency and reproducibility of this method being previously reported and confirmed [25].

Plasma levels of acetate were measured using liquid chromatography tandem mass spectrometry as previously described [26], with acetic acid-*d4* (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) used as an internal standard. Samples were first deproteinized and acetate was derived using 2-nitrophenylhydrazine, derivatives extracted by methyl *tert*-butyl ether and then injected into the liquid chromatography tandem mass spectrometry system using ACQUITY UPLC system (Waters, Milford, MA, USA), with an analytical column (AQUITY HSS T3 2.1 × 150 mm, 1.8 μm, Waters). Electrospray ionization was carried out with the API4000 (AB Sciex, Foster City, CA, USA) operating in negative ionization and multiple reaction-monitoring mode. The multiple reaction-monitoring transitions labelled for acetic acid and acetic acid-*d4* were *m/z* 194-164 and *m/z* 197-93, respectively.

2.6. *DNA extraction and microbiota analysis*

After collection from subjects at baseline and at the end of the intervention, stool samples were stored at -80°C until DNA extraction was performed. Briefly, microbial DNA was then isolated from fecal contents using QIAamp Fast DNA Stool Mini Kit (QIAGEN, Tokyo, Japan) as per the manufacturer's instructions. Polymerase chain reaction amplification was then performed targeting the V1-V2 region; forward primer (CCATCTCATCCCTGCGTGTCTCCGACTCAGNNNNNNNNNNagrgtttgatymtggctcag) and reverse primer (CCTATCCCCTGTGTGCCTTGGCAGTCTCAGtgctgcctcccgtaggagt) containing the GS Junior adapter sequences A and B, respectively, and a unique 10-bp barcode sequence for forward primer for each sample. Polymerase chain reaction amplification for 20 ng of extracted DNA was performed using Tks Gflex DNA Polymerase (Takara Bio, Inc., Shiga, Japan) under conditions of 1 minutes at 94°C, 10 cycles of 96°C for 10 seconds, 55°C for 15 seconds, and 68°C for 60 seconds, plus 25 cycles of 96°C for 10 seconds, 60°C for 15 seconds, and 68°C for 60 seconds. The amplification gave approximately 370-bp products, confirmed by agarose gel electrophoresis. Then, the

products were purified by AMPure XP magnetic beads (Beckman Coulter, Inc., CA, USA) and quantified using the PicoGreen dsDNA Assay kit (Thermo Fisher Scientific). Equal amounts of products were mixed, and sequencing was performed according to the manufacturer's instructions (GS Junior: Roche, Basel, Switzerland) as previously reported [27] with some modifications. An original pipeline was used for 16S ribosomal RNA (rRNA) analysis, which was constructed and provided by Kim et al. [27] and was built into the GS Junior PC (CentOS 6). The reference sequences for 16S rRNA analysis were created from 2 databases, the bacterial 16S rRNA sequences from the Ribosomal Database Project and the bacterial genome sequences obtained from the National Center for Biotechnology Information FTP site. The 16SrRNA sequences pyrosequenced by GS Junior, were filtered by QV (>25), amplicon length of V1-V2 region (>250 bp), and removed homopolymers (>7 bp). Read sequences with a BLAST match length of less than 90% to the reference sequences were also removed to eliminate the possibility of chimeric sequences. Reads that passed the above filters were clustered using the UCLUST program with a cutoff of 96% pairwise identity to produce operational taxonomic units. Representative sequences of each operational taxonomic unit were mapped by BLAST search to the reference sequences with a 96 % pairwise-identity cutoff and assigned to bacterial species.

2.7. *Statistical analysis*

Because no previous studies had assessed the effect of brown or white rice amazake on gut microbiota, GPower [28] was used to confirm a sample size of 40, $P < .05$ (2-sided), at the recommended power of 80%, as suitable for this study. Subject characteristics and plasma SCFA data are shown as means ± standard error of the mean (SEM), with nonparametric Mann-Whitney test used to analyze differences between WA and BA groups. MicrobiomeAnalyst [29] was used analyze differences in community composition and to calculate alpha and beta diversity within and between WA and BA groups. Briefly, data were filtered by removing very small counts using a 20% prevalence filter (for a given feature to be retained, at least 20% of its values contain at least 4 counts) and then normalized to address sparsity and ensure more meaningful comparisons. Nonparametric Mann-Whitney tests were then used to compare alpha diversity (Chao1), and beta diversity (Bray-Curtis differences) using permutational multivariate analysis of variance (PERMANOVA). Nonparametric Mann-Whitney tests were also used to assess differences in gut microbiota at major taxonomic levels and depicted using heat tree analysis. Significant differences in gut microbiota were at the species level were identified using linear discriminant analysis (LDA) effect size (LEfSe) analysis with features classified as significant using the adjusted *P* value of .05 and LDA score of 2.0. Differential abundance analysis of microbiota at the species level was performed using nonparametric Mann-Whitney tests. Spearman rank correlation method was used to analyze the relationship between the microbiome and clinical indices using the R-package, version 4.0.3, using the *cor* function, and Circlize and ComplexHeatmap from R-package to generate heat maps. MicrobiomeAnalyst was also used for other relevant statistical analyses [29]. Levels of statistical significance were set at $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$. Statistical analyses were performed in Graph Pad Prism, version 9.2 (Graph Pad Software, San Diego, CA, USA).

3. Results

At baseline, no significant differences in anthropometric or clinical parameters were observed between WA and BA groups (Table 2). Regarding biochemical parameters, level of blood glucose was significantly higher in the WA group with a mean of 112.7 ± 3.8 mg/dL compared with the BA group with a mean of 100.4 ± 2.7 mg/dL ($P = .018$). The number of subjects with T2DM was significantly higher in WA group (n = 10) versus the BA group (n = 3) ($P = .017$).

Regarding alpha and beta diversity as well as the abundance of bacteria, at the phylum, class, order, and family levels, no significant differences were observed between those allocated to the WA or BA group. However, a number of significant differences between groups at the genus and species level were observed (Figure 2). Specifically, heat tree analyses showed that the WA group exhibited a lower abundance of *Bacteroides intestinalis* ($P = .042$) and *Faecalibacterium sp DJF VR20* ($P = .036$) in comparison to the BA group (Figure 2A). In addition, an altered abundance was observed for genus *Sutterella* ($P = .011$), and the comprising species, *Sutterella wadsworthensis* ($P = .011$) as well as *Parabacteroides distasonis* ($P = .003$) and *Alistipes onderdonkii* ($P = .048$), which were elevated in the BA group in comparison to the WA group at baseline (Figure 2A). Moreover, LEfSe analysis, showing significant taxa ranked in decreasing order based on their LDA scores, further confirmed that these 5 species—*Bacteroides intestinalis, Faecalibacterium sp DJF VR20, Sutterella wadsworthensis, Parabacteroides distasonis,* and *Alistipes onderdonkii*—were significantly lower and higher in WA and BA groups, respectively (Figure 2B).

Following consumption of the test beverages after 4 weeks, no significant differences in anthropometric, clinical, or biochemical parameters were detected between the WA and BA groups (Table 3). Also, no significant differences were observed in the concentration of plasma levels of the SCFAs acetate, butyrate, propionate, or valerate between the WA and BA groups after 4 weeks or within the WA or BA groups (baseline vs 4 weeks) (Fig. 3A-D). However, within-group comparisons did reveal an increasing trend in the BA group for butyrate (baseline: 0.431± 0.670 vs 4 weeks: 0.613 ± 0.129, $P = .491$) compared with the WA group (baseline: 0.506 ± 0.076 vs 4 weeks: 0.475 ± 0.081, $P = .996$) (Fig. 3C). Similarly, within-group comparisons showed that valerate had increasing tendency in the BA group (baseline: 0.217 ± 0.032 vs 4 weeks: 0.2981 ± 0.037, $P = .298$) in comparison to the WA group (baseline: 0.243 ± 0.036 vs 4 weeks: 0.240 ± 0.030, $P = 1.00$) (Fig. 3D).

Moreover, no significant differences in alpha diversity (diversity within samples) using Chao1 (Fig. 4A) were detected between WA and BA groups after 4 weeks. Principle coordinates analysis using Bray-Curtis distances, with the percentage of variation explained by axis 1 (22.3%) and axis 2 (10.8%), also showed no significant differences in beta diversity (dissimilarity in community composition) between WA or BA subjects (Fig. 4B). Taxonomy-based abundance analyses of gut microbiota at phylum level revealed no statistically signifi-

Table 2 – – Baseline characteristics of subjects

Anthropometric and clinical parameters	WA Mean	BA Mean	P value
Age (y)	56.7 ± 2.2	58.5 ± 2.2	.722
Height (cm)	158.4 ± 1.7	159.8 ± 1.8	.578
Body weight (kg)	72.7 ± 3.2	73.4 ± 2.0	.569
BMI (kg/m^2)	28.8 ± 0.8	28.8 ± 0.7	.569
Waist circumference (cm)	95.9 ± 1.7	95.6 ± 1.9	.846
Systolic blood pressure (mmHg)	138.2 ± 2.9	136.4 ± 2.8	.673
Diastolic blood pressure (mmHg)	81.5 ± 1.8	81.4 ± 1.8	.595
Biochemical parameters			
Total cholesterol (mg/d)	205.3 ± 6.3	209.6 ± 6.6	.379
HDL-cholesterol (mg/dL)	53.0 ± 2.9	53.0 ± 2.8	.995
LDL-cholesterol (mg/dL)	120.5 ± 6.1	132.1 ± 6.3	.344
Triglycerides (mg/dL)	180.4 ± 33.1	134.5 ± 10.0	.984
Glucose (mg/dL)	112.7 ± 3.8	100.4 ± 2.7	.018*
HbA1c (%)	6.3 ± 0.1	5.9 ± 0.1	.059
Insulin (μIU/mL)	10.5 ± 11.8	10.0 ± 1.0	.615
HOMA-IR	2.9 ± 0.5	2.6 ± 0.3	.836
Comorbidities (N, %)			
High blood pressure	17 (89%)	20 (95%)	0.596
Dyslipidemia	16 (84%)	18 (86%)	>0.999
Type 2 diabetes mellitus	10 (53%)	3 (14%)	0.017*

Data indicate mean values ± standard errors of the mean. All data were analyzed using nonparametric Mann-Whitney test to compare WA and BA groups. WA, n = 19; BA, n = 21; *P < .05.
Abbreviations: BA, brown rice amazake; BMI, body mass index; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; HOMA-IR, Homeostatic Model Assessment for Insulin Resistance; LDL, low-density lipoprotein; WA; white rice amazake.

Table 3 – – Characteristics of subjects at 4 weeks

Anthropometric and clinical parameters	WA Mean	BA Mean	P value
Body weight (kg)	72.8±3.2	73.6±2.1	.507
BMI (kg/m^2)	28.8±0.8	28.8±0.7	.606
Waist circumference (cm)	95.8±1.5	95.8±1.9	.857
Systolic blood pressure (mmHg)	137.6±3.2	133.9±2.6	.386
Diastolic blood pressure (mmHg)	81.5±1.8	80.9±1.8	.614
Biochemical parameters			
Total cholesterol (mg/d)	200.6±4.5	205.2±4.9	.386
HDL-cholesterol (mg/dL)	52.7±3.2	51.0±2.5	.663
LDL-cholesterol (mg/dL)	118.7±4.5	128.4±4.3	.100
Triglycerides (mg/dL)	161.1±21.2	154.9±20.1	.984
Glucose (mg/dL)	111.6±4.8	102.2±3.0	.092
HbA1c (%)	6.3±0.1	5.9±0.1	.063
Insulin (μIU/mL)	9.3±1.1	10.1±1.0	.634
HOMA-IR	2.5±0.3	2.6±0.3	.888

Data indicate mean values ± standard error of the mean. All data were analyzed using nonparametric Mann-Whitney test to compare WA and BA groups. WA, n = 19; BA, n = 21; *P < .05.
Abbreviations: BA, brown rice amazake; BMI, body mass index; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; HOMA-IR, Homeostatic Model Assessment for Insulin Resistance; LDL, low-density lipoprotein; WA; white rice amazake.

cant differences between the WA and BA groups (Fig. 4C). Importantly, significant differences were observed at the family, genus, and species levels between the WA and BA groups, with heat tree analysis graphically highlighting these differences (Fig. 4D). Specifically, an altered abundance of bacteria from the family Porphyromonadaceae was observed in BA subjects versus WA subjects (P = .013) and similarly for the comprising genera *Parabacteriodes* (P = .011). Compared with the BA group, the WA group exhibited a significantly lower abundance of *Butyricicoccus* (P = .012) genus and comprising species *Butyrate producing bacterium A2 207* (P = .012). A significantly different abundance of bacteria in the WA and BA groups from the *Sutterella* genus (P = .001) and *Sutterella wadsworthensis* at the species level (P = .001) were also observed. Moreover, the WA and BA groups differed in the abundance of *Lactobacillales bacterium DJF B280* (P = .005), and *Firmicutes bacterium DJF VP44*

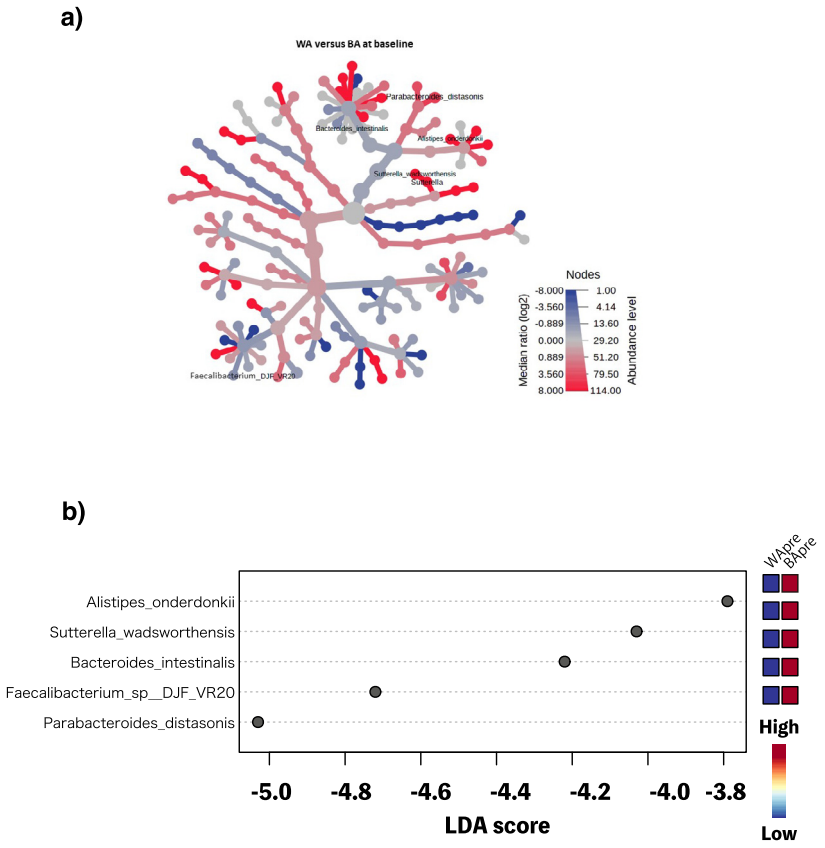

Fig. 2 – Composition of gut microbiota in WA and BA subjects at baseline. (A) Heat tree analysis showing taxonomic differences between WA and BA microbial communities with significant alterations in taxa shown by name of the bacteria at the corresponding node. Position and size of nodes indicate hierarchical structure of taxa and colors of nodes and branches indicate either an increase (red) or decrease (blue) in abundance level based on the log2 ratio of median abundance. (B) Linear discriminant analysis effect size (LEfSe) between WA and BA groups with significant taxa graded in decreasing order by LDA score and heatmap showing corresponding taxa that are either higher (red) or lower (blue). Data were analyzed using nonparametric Mann-Whitney tests to compare WA and BA groups at baseline. WA, n = 17; BA, n = 17. *$P < .05$. BA, fermented brown rice beverage; WA, fermented white rice beverage.

($P = .038$), both from the Firmicutes phylum. LEfSe analysis further highlighted significant differences in *Butyrate producing bacterium A2 207*, *Sutterella wadsworthensis*, and *Firmicutes bacterium DJF VP44*, which were all increased and decreased in the BA and WA groups, respectively (Fig. 4E). Differential abundance analyses further showed that these species clearly demonstrated a significant elevation in the BA group in comparison to WA group (Fig. 5A-D).

When comparing baseline and 4-week microbiota data within groups (baseline vs 4 weeks), no significant differences in alpha diversity (diversity within samples) using Chao1 (**Supplementary Figure S1a and b**) were observed within either WA or BA groups. Principle coordinates analysis using Bray-Curtis distances, with the percentage of variation explained by axis 1 (23.7%) and axis 2 (13.3%), in the WA group (**Supplementary Figure S1c**) and (18.3%) and axis 2 (12.3%) in the BA group (**Supplementary Figure S1d**), also showed no significant differences at baseline and 4 weeks. Taxonomy-based abundance analyses of gut microbiota at phylum level revealed no statistically significant differences within WA (**Supplementary Figure S2a**) and BA (**Supplementary Figure S2b**) groups at baseline and 4 weeks. In contrast, heat map analysis showed a significant alteration of the species *Flavonifractor plautii* ($P = .026$) in the WA group after 4 weeks in comparison to baseline (**Supplementary Figure S2c**), with differential abundance analyses visually showing an increase of this species within the WA group at 4 weeks (**Supplementary Figure S2d**).

To explore the relationship between the gut microbiota and components of the metabolic syndrome, Spearman rank correlation coefficient method was used to assess the correlation between each subject's gut microbiota at the species level and anthropometric, clinical, and biochemical indices related to the metabolic syndrome (body weight, body mass index, waist circumference, systolic blood pressure, diastolic blood pressure, total cholesterol, HDL-C, LDL-cholesterol, triglycerides, glucose, HbA1c, insulin, and Homeostatic Model Assessment for Insulin Resistance). Significant correlations (correction $r > 0.4$ or $r < -0.4$, $P < .05$) were observed among 41 taxa and the 13 clinical indices (Fig. 6), with the abundance level of only 3

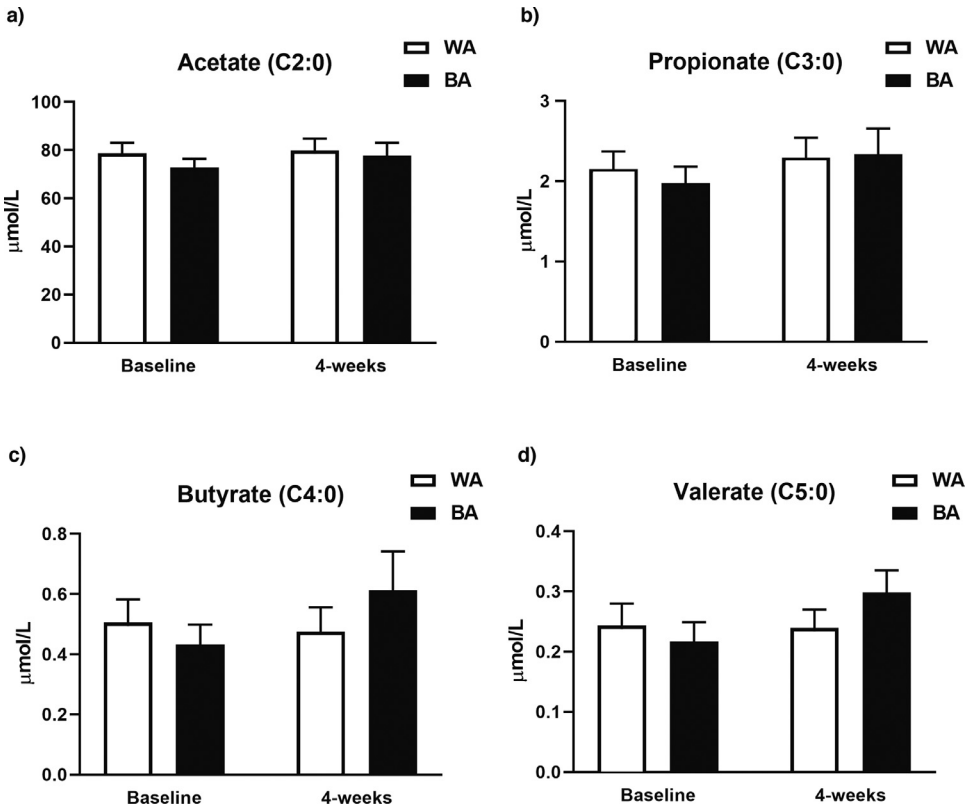

Fig. 3 – Plasma SCFA profile of subjects comparing WA or BA groups at 4 weeks and within WA and BA groups (baseline and 4 -weeks). (A-D) Individual plasma SCFA concentrations (acetate, propionate, butyrate, and valerate, respectively). Data indicate means and standard errors of the mean (SEM). All data were analyzed using 2-way ANOVA followed by Tukey post hoc test. WA, n = 16; BA, n = 19. *P < .05. BA, fermented brown rice beverage; SCFA, short-chain fatty acid; WA, fermented white rice beverage.

species differing significantly between the WA and BA groups. Interestingly, a significantly negative correlation observed between *Sutterella wadsworthensis* and blood glucose ($P = .032$) and significantly negative correlations also observed between *Lactobacillales bacterium DJF B280* and triglyceride level ($P = .006$) as well as *Butyrate producing bacterium A2 207* and triglyceride level ($P = .001$). A strong positive correlation was found between *Lactobacillales bacterium DJF B280* and HDL-C ($P = .037$) and a more moderate positive correlation for *Butyrate producing bacterium A2 207* and LDL-C ($P = .046$).

4. Discussion

In line with our hypothesis, consumption of BA, as opposed to WA, was able to significantly modulate the gut microbiota and contributed to an increasing trend in the level of plasma butyrate and valerate. Importantly, ingestion of BA as opposed to WA most notably impacted gut microbiota composition at the species level. Interestingly, an increase in *Butyrate producing bacterium A2 207* from the *Butyricicoccus* genus was observed in BA subjects versus WA subjects. As the name suggests, this species of bacteria is capable of producing butyrate, with reduced levels of butyrate-producing bacteria observed in individuals with a variety of metabolic and chronic diseases [30, 31]. Although we did not observe a significant difference in plasma butyrate levels between groups, we did observe an increasing tendency in the BA group. In addition, a greater abundance of *Lactobacillales bacterium DJF B280,* often found in fermented foods, as well as *Firmicutes bacterium DJF VP44,* were significantly elevated in the BA group in comparison to the WA group. These species, belonging to Clostridium cluster IV, have an indispensable role in creating immune tolerance and lowering levels of inflammatory cytokines and increasing regulatory T-cell induction via both SCFA-dependent and independent mechanisms [32, 33]. Interestingly, *Lactobacillales bacterium DJF B280* and *Firmicutes bacterium DJF VP44* as well as *Butyrate producing bacterium A2 207* are capable of producing butyrate, lactate, and ethanol [18]. Lactate can also act as a substrate for other bacteria that produce butyrate and propionate [34], and production of valerate is also possible via the coupling of propionate with ethanol [18]. In this context, an increased abundance of these bacterial species in the BA group may have contributed to the tendency of plasma butyrate and valerate levels to increase observed in the BA group after 4 weeks.

The potential mechanisms as to how to BA may characteristically modulate the gut microbiota may be due to a number of beneficial components. First, fiber, which is higher in BA compared with WA, can lead to an increase in abundance of fiber-degrading bacteria, capable of producing SCFAs such as those species seen elevated in the BA group [35]. In addition,

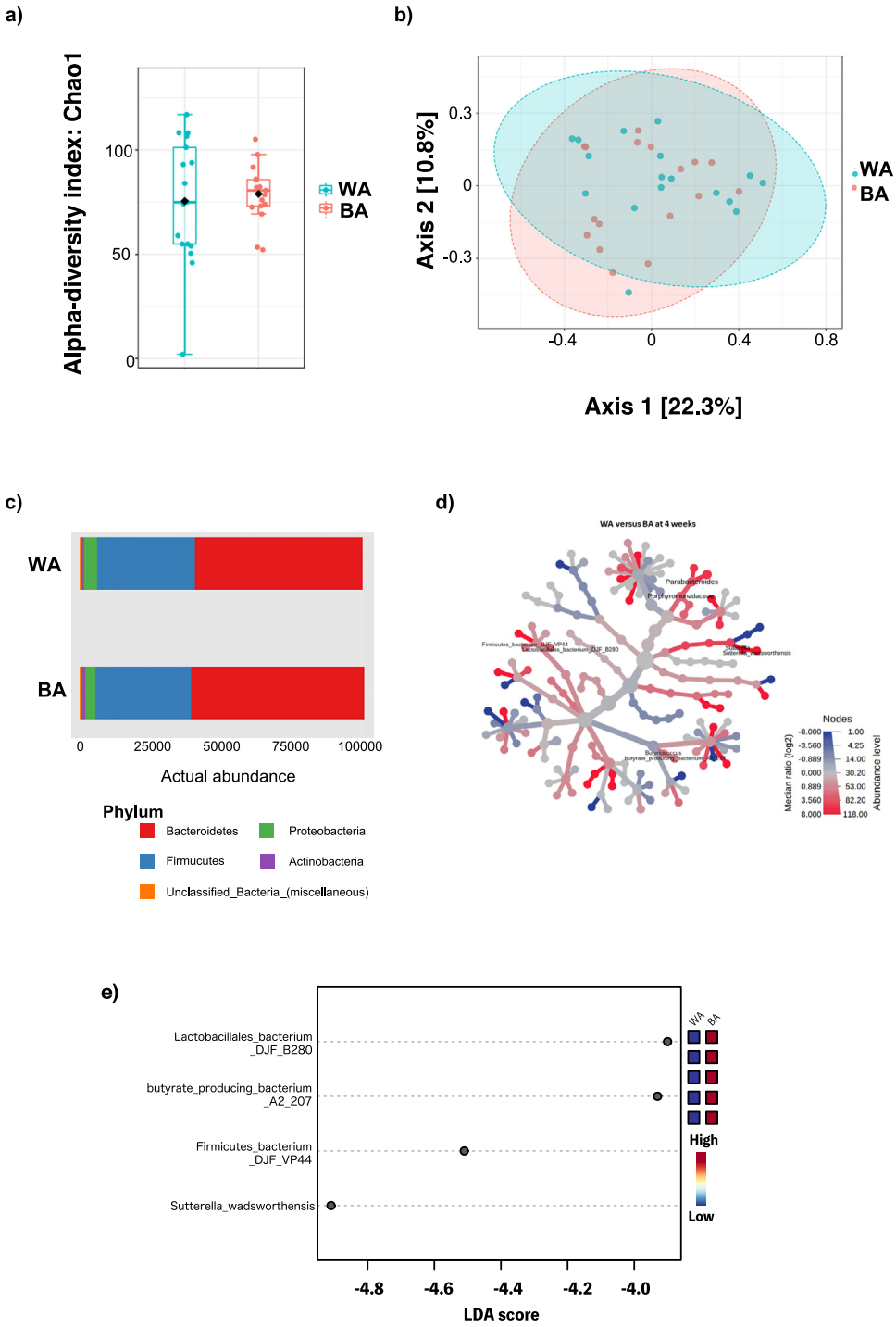

Fig. 4 – Diversity and composition of gut microbiota in WA and BA subjects at 4 weeks. Box plot showing alpha diversity as measured by (A) chao1 index, estimating taxa richness by accounting for low abundance; (B) principal coordinate analysis of gut microbiota composition depicting beta diversity and using Bray-Curtis distance matrix highlighting differences between groups; (C) actual abundance of major phyla composition; and (D) heat tree analysis showing taxonomic differences between WA and BA microbial communities with significant alterations in taxa shown by name of the bacteria at the corresponding node. Position and size of nodes indicate hierarchical structure of taxa and colors of nodes and branches indicate either an increase (red) or decrease (blue) in abundance level based on the log2 ratio of median abundance. (E) Linear discriminant analysis effect size (LEfSe) between WA and BA groups with significant taxa graded in decreasing order by LDA score and heatmap showing corresponding taxa that are either higher (red) or lower (blue). Data were analyzed using nonparametric Mann-Whitney tests to compare differences in alpha diversity and microbiota composition (heat tree, LEfSe). Beta diversity was analyzed using permutational multivariate analysis of variance (PERMANOVA). WA, n = 17, BA, n = 17. *P < .05. BA, fermented brown rice beverage; WA, fermented white rice beverage.

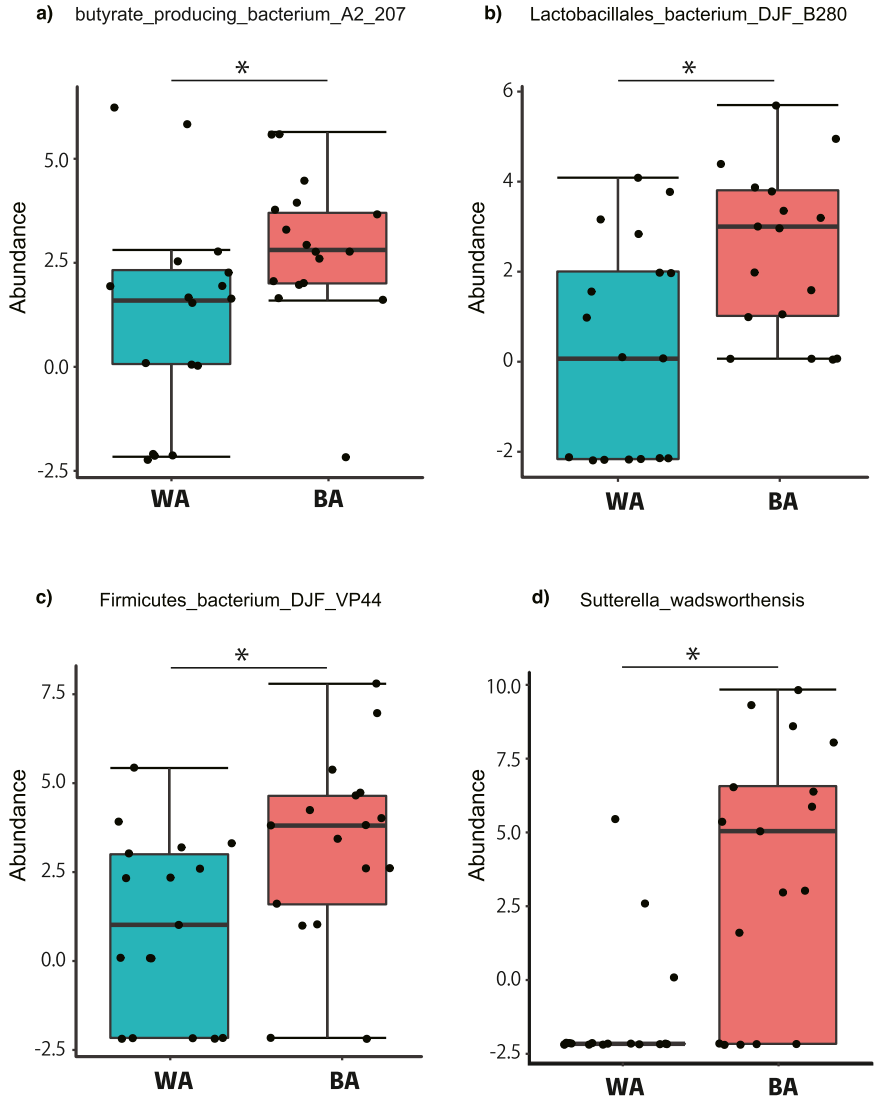

Fig. 5 – (A-D) Differential abundance analysis (log-transformed count) of selected gut microbiota species showing significant differences between WA and BA groups. Data indicate means and their standard errors of the means (SEM). Data were analyzed using nonparametric Mann-Whitney tests. WA, n = 17; BA, *n* = 17. *P < .05. BA, fermented brown rice beverage; WA, fermented white rice beverage.

BA abundantly contains γ-oryzanol, whereas WA does not so much. Intriguingly, the main metabolite of γ-oryzanol, ferulic acid, a phenolic compound that possesses potent antioxidant and anti-inflammatory activity [36], relies on microbial transformation to be converted to its bioactive form by species such as those from the *Clostridium cluster IV*, which were seen elevated in the BA group [32].

When examining differences within groups at baseline and after 4 weeks, no apparent differences were observed in the BA group. However, ingestion of WA resulted in somewhat negative alterations to gut microbiota. Specifically, *Flavonifractor plautii*, a flavonoid-degrading bacteria [37], was reduced at 4 weeks in comparison to baseline levels in the WA group. Although data on the nutrient intake of subjects were not obtained, there may be a possibility that a reduction in this species could occur as a result of the fiber and flavonoid-containing foods eaten in the subject's usual diet at baseline, being replaced with WA, which instead contains minimal amounts of phytochemicals and fiber. The lack of differences observed in the BA group at 4 weeks versus baseline may have been partially because of the acute nature of the intervention period, with more beneficial changes in gut microbiota likely observed after a longer duration of daily BA ingestion [38].

Importantly, at baseline, although differences were not observed between groups for most subject characteristics, a significantly greater number of subjects with T2DM, and thus correspondingly higher levels of blood glucose, were observed in the WA group. In addition to this, a significantly greater abundance of bacterial species—*Alistipes onderdonkii*, *Parabacteroides distasonis*, *Bacteroides intestinalis*, and *Faecalibacterium sp DJF VR20*–were observed in the BA group at baseline and abundance of *Sutterella wadsworthensis* was found elevated in the BA group both at baseline and after the 4-week intervention in comparison to WA group. Although these findings represent

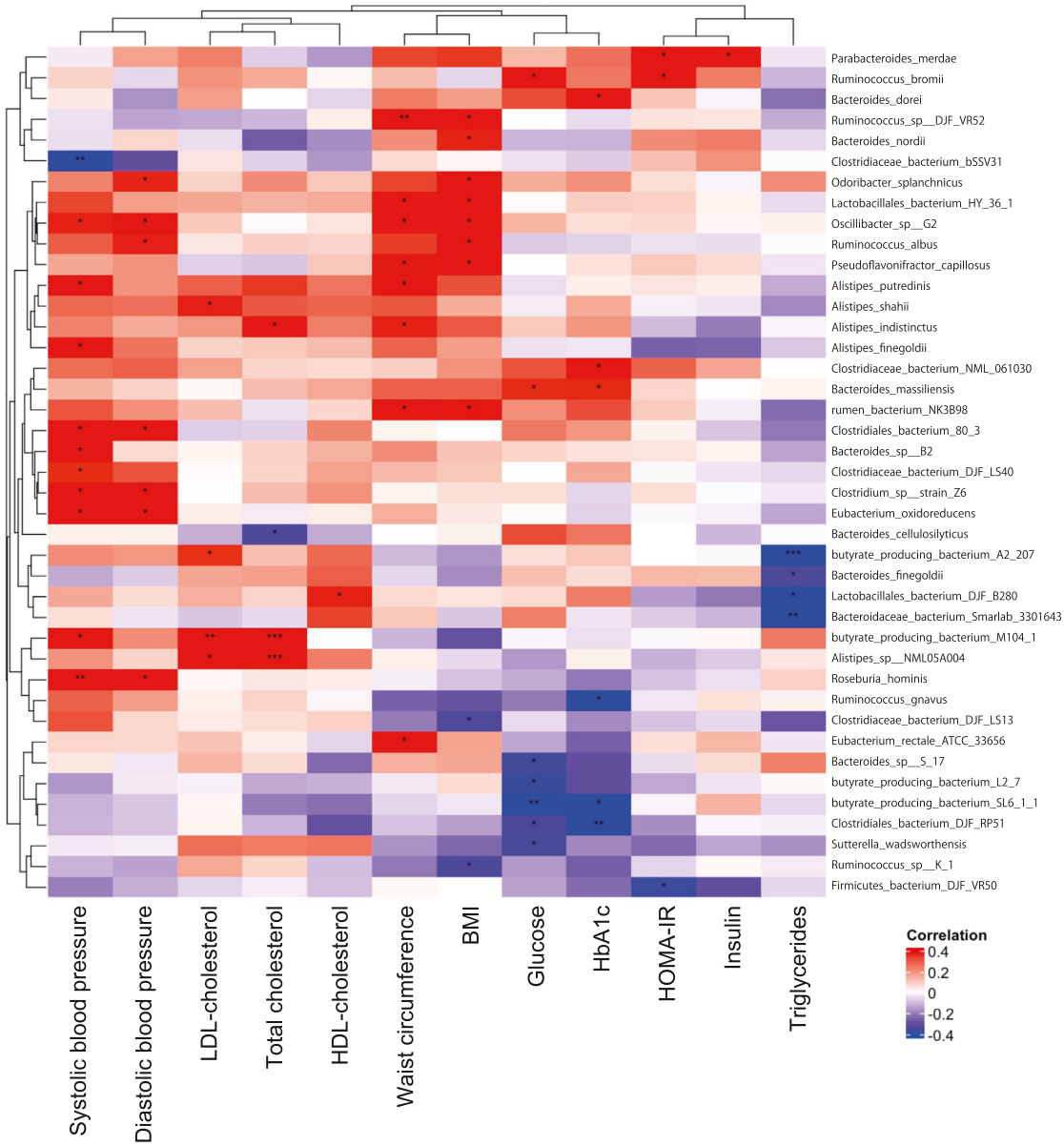

Fig. 6 – Heat map of Spearman correlation analysis highlighting significant correlations of gut microbial species and selected anthropometric, clinical, and biochemical indices of study subjects at 4 weeks. Significant correlations (correction $r > 0.4$ or $r < -0.4$) are shown as blue (negative) or red (positive). n = 34 (WA and BA). *$P < .05$, **$P < .01$ and ***$P < .001$. BA, fermented brown rice beverage; WA, fermented white rice beverage.

nonnegligible limitations to the present study, with the difference in gut microbiota possibly from the differences in number of subjects with T2DM between groups, this likely does not represent the only factor involved in causing these disparities. First, only differences in the bacteria *Sutterella wadsworthensis* was observed at both baseline and remained at the end of the 4-week intervention between the WA and BA groups. This raises the question that the lower abundance of *Sutterella wadsworthensis* in the WA group in comparison to BA group could be impacted by the presence of T2DM. Intriguingly, a reduced abundance of *Sutterella* genus (containing *Sutterella wadsworthensis*) has been observed in subjects with prediabetes and newly diagnosed diabetes and T2DM in comparison to healthy controls [39]. Also, a number of other variables that were not measured in the present study, such as habitual diet, exercise, and sleep, may have also contributed to the differences in gut microbiota observed between groups [38, 40, 41].

At the end of the 4-week intervention, no significant differences in anthropometric, clinical, or biochemical parameters were observed between WA and BA groups, which again is likely a result of the relatively brief intervention period. However, given that a number of significant changes in the gut microbiota were observed between WA and BA groups, this raises the question as to whether changes in gut microbiota may precede changes in, or be correlated with, various metabolic markers. To explore this, we undertook correlation analyses

to identify if there were any significant relationships between the gut microbiota and anthropometric, clinical, and biochemical parameters of interest in the context of the metabolic syndrome.

We found that *Sutterella wadsworthensis*, which was elevated in the BA group at baseline and 4 weeks, was negatively correlated with blood glucose. These findings are in line with previous studies reporting a reduced abundance of *Sutterella* in subjects with T2DM [39, 42]. Interestingly, an increase in *Sutterella* and improvement in glucose metabolism has been reported after Roux-en-Y gastric bypass surgery in diabetic rats [42]. Significantly negative correlations were also observed between *Lactobacillales bacterium DJF B280* and *Butyrate producing bacterium A2 207* and triglyceride level. These findings are in line with a large population-based cohort study of 893 subjects that found that gut microbiota from the Clostridiaceae family, containing both *Lactobacillales bacterium DJF B280* and *Butyrate producing bacterium A2 207*, which were elevated in the BA group, were negatively correlated with triglyceride levels [43]. Our study also found that *Lactobacillales bacterium DJF B280* was significantly correlated with HDL-C level. In particular, SCFA-producing bacteria from Clostridiaceae, containing *Lactobacillales bacterium DJF B280,* have been shown to be positively correlated with HDL-C [43] and demonstrate overall favorable effects on cholesterol metabolism in both animals and humans [44]. Although a positive correlation was observed between *Butyrate producing bacterium A2 207* and LDL-C in our study, the majority of published literature points toward a beneficial effect of this species and other butyrate-producing bacteria in lipid metabolism [43–45].

In conclusion, consumption of BA in comparison to WA, resulted in beneficial changes to the gut microbiota in subjects with MetS. These results support recent research regarding the beneficial effects of whole grains and fermented foods such as BA on the gut microbiota. Longer term interventional studies are required to further strengthen the impact of BA on the gut microbiome and the potential impact on markers of metabolic health.

Declaration of Competing Interest

There are no conflicts of interest to declare.

Author contributions

Yukari Akamine: Formal Analysis, Writing Original Draft, Writing – Review & Editing, and Visualization. Jasmine F. Millman: Formal Analysis, Writing Original Draft, Writing – Review & Editing, and Visualization. Tsugumi Uema: Formal Analysis, Writing Original Draft, Writing – Review & Editing, and Visualization. Shiki Okamoto: Writing – Review & Editing and Supervision. Masato Yonamine: Investigation and Project Administration. Moriyuki Uehara: Investigation and Project Administration. Chisayo Kozuka: Methodology and Investigation. Tadashi Kaname: Software, Resources, and Data Curation. Michio Shimabukuro: Conceptualization and Project Administration. Kozen Kinjo: Formal Analysis, Investigation, and Resources. Masayo Mitsuta: Formal Analysis, Investigation, and Resources. Hirosuke Watanabe: Formal Analysis, Investigation, and Resources. Hiroaki Masuzaki: Conceptualization, Methodology, Writing – Review & Editing, Supervision, Project Administration, and Funding Acquisition. All authors read and approved the final manuscript.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.nutres.2022.03.013.

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