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In vitro colon fermentation of a traditional fermented food using stool from consumers and non-consumers

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Traditional fermented foods (TFFs) spark debates about their potential health benefits as much is still unclear. Mostly conjectured as functional or living foods, TFFs are produced by live organisms through spontaneous or controlled fermentation processes. Here, we assessed the effect of exposure to Mabisi, a traditional Zambian fermented dairy food product, on the gut microbiota and production of short-chain fatty acids (SCFAs) in stool samples of consumers and non-consumers of Mabisi. We hypothesize that non-consumers of Mabisi will exhibit greater shifts in the gut microbiota composition and a more pronounced increase in SCFA production compared to Mabisi consumers. Stool samples of consumers and non-consumers of Mabisi were exposed to three treatments: (1) Mabisi, (2) fructooligosaccharides (FOSs) as a positive control, and (3) sterile water as a negative control. Treatments were digested using the *in vitro* INFOGEST static digestion model protocol 2.0 before anaerobic incubation for 24 h with the stool of consumers and non-consumers of Mabisi. We sequenced the hypervariable region (V3–V4) of the 16S rRNA gene to determine the microbial communities. We measured SCFA production as a proxy for gut microbiota functionality. Mabisi supplementation increased *Pediococcus* in both consumers and non-consumers of Mabisi compared to sterile water. After treatment with Mabisi, the gut microbiota of consumers showed greater resilience, with limited changes in community composition compared to non-consumers, as indicated by beta diversity (Mabisi consumers: R^2 = 0.07, p-adjusted = 0.375; Mabisi non-consumers: R^2 = 0.08, p-adjusted = 0.05) relative to their respective negative controls. Non-consumers were associated with higher production of SCFAs, including acetate, butyrate, formate and succinate, compared to Mabisi consumers. In conclusion, Mabisi has the potential to modulate *in vitro* gut microbiota by increasing beneficial bacteria and the production of SCFAs, with a particularly strong effect in non-consumers.

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Introduction

Traditional fermented foods (TFFs) spark debates among scientists relating to their potential health benefits to humans. Studies conducted on the bacterial composition of TFFs reveal consistent dominance of Firmicutes and Actinobacteria phyla, also in the case of spontaneous fermentation, a way through which TFFs are typically produced.[1] Beneficial bacterial genera from these two phyla may include *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Pediococcus*, and *Bifidobacterium*.[2] After consumption of TFFs, certain strains of bacteria may end up in the colon and potentially act as probiotics. Probiotics fuel the host gut mucosa cells and may contribute to health by inhibiting the growth of pathogenic microorganisms in the gut. Another possibility conjecturing the health benefits of TFFs is the high abundance of specific metabolites generated during the fermentation process, including short-chain fatty acids (SCFAs), diacetyl, hydrogen peroxide, acetaldehyde, ethanol, bacteriocins, and reuterin.[3] These metabolites may have protective properties for the host gut mucosa. A high concentration of metabolites could alter the gut environment, thereby selectively favouring the growth of beneficial bacteria within the colon and thus impacting host health positively. Generally, through known and unknown mechanisms, most TFFs are reported to modulate the immune system, reduce glycosylated haemoglobin (HbA1C) in diabetes mellitus type two,[4] reduce the production of pro-inflammatory metabolites,[5] and reduce

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undesirable symptoms in individuals with inflammatory bowel diseases.[6]

Regular intake of TFFs such as Kefir, Sauerkraut, Chibwantu, Kimchi, Munkoyo, Ergo, and Mabisi may result in a resilient and stable gut microbiota that benefits the host's health. In a systematic review on the effect of Kefir consumption on human health, wider health claims are documented that show the potential of TFFs in modulating the gut microbiota.[7] Here, resilience and stability would imply a gut microbiota that will not easily change bacterial composition upon exposure to external pressures.[8] Therefore, we expect that the gut microbiota of non-consumers of TFFs will be less resilient and more susceptible to changes in bacterial composition when exposed, for instance, to a TFF.

An example of a TFF widely studied is Mabisi.[9–11] Mabisi is a traditional fermented milk product widely consumed across Zambia. It is produced through spontaneous fermentation of raw cow's milk under non-pasteurized conditions using various vessels such as calabashes or plastic containers, over a period of 1–3 days at ambient temperatures. The fermentation is driven by naturally occurring lactic acid bacteria and yeasts, leading to the development of unique sensory, microbial, and nutritional properties. Previous studies have identified diverse microbial communities in Mabisi, including genera such as *Lactobacillus*, *Leuconostoc*, and *Streptococcus*, some of which are known to have probiotic potential.[12,13] Despite its widespread consumption and cultural significance, Mabisi remains under-characterized scientifically. In particular, the effects of regular consumption of Mabisi on gut microbiota composition and short-chain fatty acid production are underexplored. Studies by Alekseeva,[14] Moonga[1] and Chileshe[15] report bacterial communities in Mabisi that could have probiotic potential. Investigating whole Mabisi for its potential to promote resilience and stability among consumers is important for promotion of its consumption, especially vulnerable populations such as children aged under 48 months.

Studying variations in the gut microbiota *via in vivo* methods is restricted for ethical and safety reasons. Therefore, *in vitro* digestion models[16–18] have been developed to study the effects of food on the microbiome and related metabolite production.[16–18] Innovation of digestion models ranges from complex automated dynamic models to simple static models, all aiming to mimic human and animal digestion. The primary purpose of these models is to simulate the physiological processes in human and animal digestion accurately for the questions at hand. Protocols validated by a consortium of digestion scientists known as the INFOGEST static digestion and fermentation model (hereafter the INFOGEST model) are highly recommended for studying the characteristics of food matrices.[17–19] The INFOGEST model may be used to complement the outcomes of advanced dynamic computerized models such as SHIME[20] and TIM.[21]

In this paper, we have used the INFOGEST model to assess the effect of exposure to a TFF, Mabisi, on the gut microbiota extracted from consumers and non-consumers of Mabisi, and how this relates to the production of SCFAs. We hypothesize that non-consumers of Mabisi will exhibit greater shifts in the gut microbiota composition and a more pronounced increase in SCFA production compared to Mabisi consumers. This hypothesis is based on an ecological principle suggesting that non-consumers have not yet been modulated by Mabisi exposure and thus may undergo more pronounced shifts in the gut microbiota composition and SCFA production as they adapt to utilize a resource, Mabisi, with modulation potential.[22]

Methods and materials

Stool samples used in this experiment were obtained from children who participated in a baseline survey conducted as part of an earlier study in Mazabuka District, Southern Province, Zambia.[23]

Experiment setup

Stool samples of consumers and non-consumers of Mabisi were exposed to the following three treatments: (1) Mabisi, (2) fructooligosaccharides (FOS) as a positive control, and (3) sterile water as a negative control. Treatments were digested using the *in vitro* INFOGEST static digestion model protocol 2.0[17–19] before anaerobic incubation for 24 h with the stool of consumers and non-consumers of Mabisi. The colon digestion protocol (also known as the fermentation protocol) simulates the colon, following work by Pérez-Burillo and colleagues.[19] Donors of stool material (C1 to C6) were children (aged 6 to 12 months) who participated in our previous unpublished study and had not been exposed to antibiotics in the last 30 days. Using a questionnaire to document their Mabisi intake, the children were classified as either Mabisi consumers or non-consumers and three biological stool replicates were sampled. For each biological replicate, three technical replicates were used per treatment. At the end of the experiment, we extracted genomic DNA for bacterial composition analysis and the supernatant for SCFA analyses (Fig. 1).

Ethical approval was granted by the Tropical Diseases Research Center Ethics Review Board in Zambia under the IRB registration number: 00002911, TRC/C4/02/2021. The Zambia National Health Research Board provided permission to access the research site. Parents or caregivers signed informed consent prior to participation in the study. This study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki of the World Medical Association.

Materials

We used the following chemicals and enzymes: soluble potato starch (S2630, Sigma-Aldrich), 3,5-dinitrosalicylic acid (DNS) (210-204-3, Sigma-Aldrich), sodium phosphate buffer (P4922, Sigma-Aldrich), sodium potassium tartrate tetrahydrate (S2377, Sigma-Aldrich), maltose standard (M5885, Sigma-Aldrich), hydrochloric acid (H1758, Sigma-Aldrich), sodium hydroxide (S58891, Sigma-Aldrich), trichloroacetic acid (T6399, Sigma-Aldrich), sodium taurodeoxycholate solution (904236, Sigma-Aldrich), tributyrin (T8626, Sigma-Aldrich), *p*-toluene-

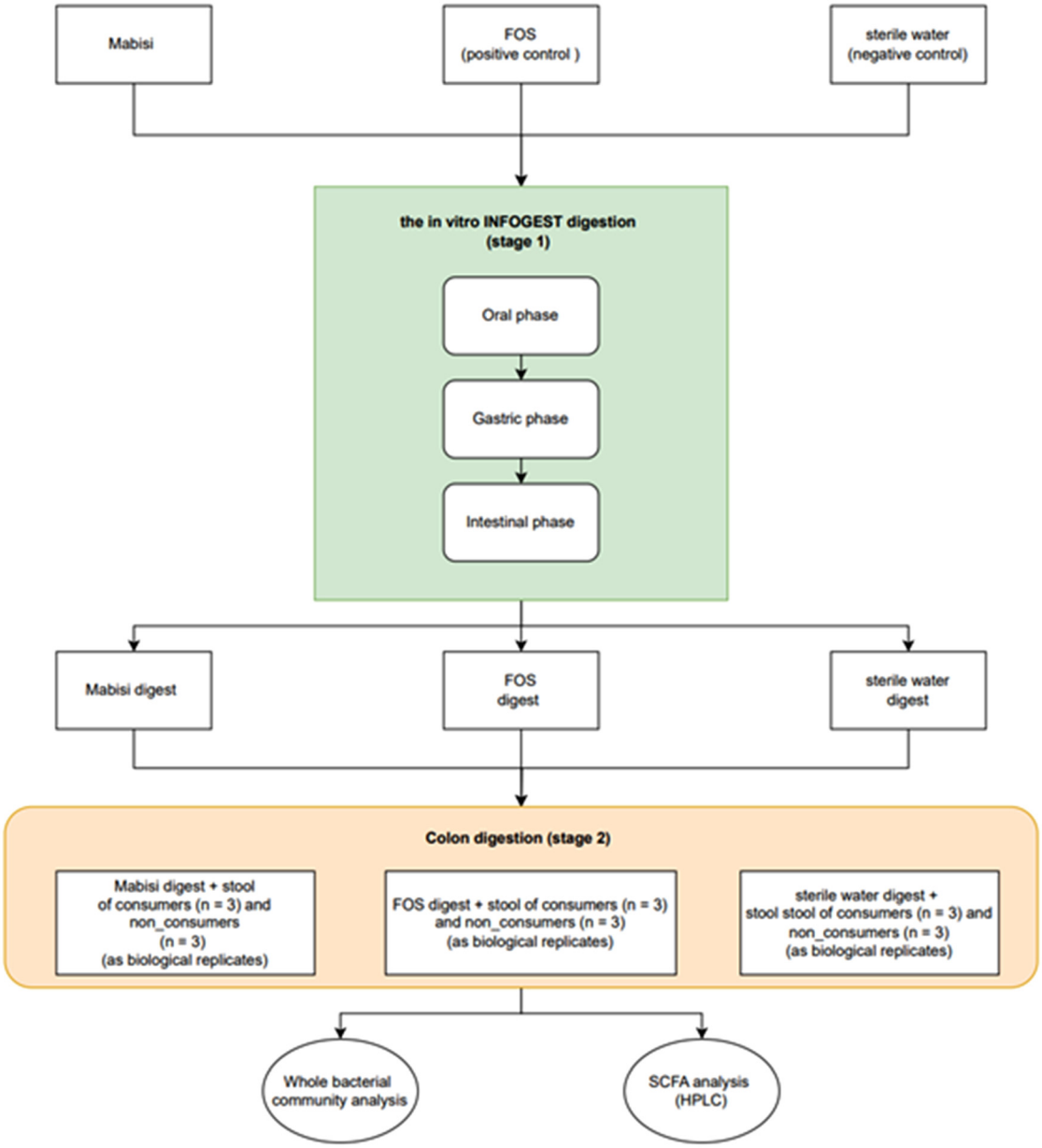

Fig. 1 A flowchart illustrating the experimental setup, which consists of two stages of digestion. Stage 1 involves *in vitro* INFOGEST static digestion of treatments (Mabisi, FOS as a positive control, and sterile water as a negative control). Stage 2 focuses on colon digestion, where treatments were incubated with stool slurry (gut microbiota) from both Mabisi consumers and non-consumers. At the end of the experiment, colon digestion contents were sampled for whole bacterial community profiling and SCFA analysis.

sulfonyl-L-arginine methyl ester-TAME (T4626, Sigma-Aldrich), bovine blood haemoglobin (H2500, Sigma-Aldrich), porcine pepsin (P6887, Sigma-Aldrich), human salivary amylase (A1031, Sigma-Aldrich), porcine pancreatin (P7545, Sigma-Aldrich), rabbit gastric extract powder (RGE25-Lipolytech), phosphocreatine disodium salt hydrate (ED2SC, Sigma-Aldrich), zinc sulfate heptahydrate (Z0251, Sigma-Aldrich), fructooligosaccharides from chicory (F8052, Sigma-Aldrich), peptone from potatoes (83059, Sigma-Aldrich), and standards of acetate, propionate, isobutyrate, butyrate, formate and lactate, all supplied by Sigma-Aldrich.

Processing of Mabisi

Full-fat UHT milk from a local supermarket in the Netherlands was used to prepare Mabisi. Mabisi (1 mL from a previous field study)[1] was added to 500 mL of full-fat milk and then incubated at 28 °C for 48 h to obtain the white thick fermented Mabisi liquid for the experiment.

In vitro INFOGEST static digestion (stage 1)

Sample digestion was carried out according to the *in vitro* INFOGEST static digestion protocol 2.0 with the following slight

adjustments for the oral, gastric and intestinal phases. For the oral phase, Mabisi (5 mL) was mixed with simulated salivary fluid (SSF) (4.5 mL) in a 50 mL centrifuge tube. A predetermined concentration of salivary amylase was added to attain an activity of 75 U mL^{-1}. The oral phase mixture was then adjusted to 10 mL using sterile water. Next, the pH of the oral phase mixture was adjusted to 7.0 by the addition of NaOH and the mixture was incubated at 37 °C for 2 min. In the gastric phase, 9 mL of simulated gastric fluid (SGF) was mixed with a 10 mL oral phase mixture. Pepsin (0.7 mL) dissolved in HCl was added to obtain 2000 U mL^{-1} pepsin activity. Then, gastric lipase (0.3 mL) from the rabbit gastric extract was added to obtain 60 U mL^{-1} activity. The pH of the solution was adjusted to 3.0 using HCl, after which the gastric mixture was marked up to 20 mL, followed by incubation at 37 °C for 2 h. Finally, in the intestinal phase, a small intestine mixture was prepared by adding 15 mL of simulated intestinal fluid (SIF) to 20 ml gastric phase mixtures, followed by the addition of 13.37 mg mL^{-1} pancreatin and bile salts (10 mM) and pH adjustment to 6.8. The final total volume for the intestine mixture was adjusted to 40 mL by adding sterile water before incubating at 37 °C for 2 h.

In vitro colon digestion of Mabisi, FOS and sterile water (stage 2)

Handling of stool samples before the experiment. Storage and processing of stool samples before the experiments were as described in our baseline study.

Preparation of stool slurry. Separately, stool material from each fresh sample of consumers and non-consumers of Mabisi was prepared as 32% (w/v) stool slurry by dissolving 32 g in 100 mL (100 mM) phosphate buffer and adjusting the pH to 7.0. Each stool slurry aliquot was vortexed and centrifuged at 16 000 rpm to obtain solid debris and supernatant fractions. From the supernatant, 2 mL of stool slurry was used as a stool inoculant for colonic digestion.

Processing of Mabisi, FOS and sterile water as intestinal phase digests. First, we centrifuged SIF contents at 16 000 rpm to obtain solid and supernatant fractions. We collected 10% of the supernatants equivalent to solids or semi-solids (0.5 g of solid and 0.62 mL of supernatant fractions). The supernatant liquid added represented the physiological fluid passed to the large intestine from the small intestine.

Processing of the colonic digestion medium. The colonic digestion medium (1000 mL) was prepared by mixing peptone (15 g L^{-1}) and 50 mL of reductive solution per litre of peptone and then autoclaved. A reductive solution was prepared by dissolving 312 mg of cysteine and 312 mg of sodium sulfide in 100 mL. After cooling, 1.25 mL of filtered resazurin (0.1% w/v) was added, after which the volume was marked up to 1000 mL.

Colonic digestion of Mabisi, FOS and sterile water. Using a 50 mL centrifuge tube, Mabisi, FOS and sterile water intestinal phase digests consisting of 0.5 g of solid and 0.62 mL of supernatant fractions, 7.5 ml of colon digestion medium and 2 mL of stool slurry were mixed for colon digestion. All colonic mixture samples were anaerobically incubated at 37 °C for 24 h.

Sampling for DNA and short-chain fatty acids from the colonic digestion phase. At the end of the incubation period, colon-digested samples were immediately submerged in ice-cold water for 5 min and then stored at −20 °C to stop further microbial activity. Before analysis, samples were thawed and centrifuged at 16 000 rpm to obtain a supernatant for SCFA extraction and a solid fraction to determine bacterial genomic DNA. We used 1.5 ml of supernatant for short-chain fatty acid analysis by High-Performance Liquid Chromatography (HPLC) (Thermo Fisher Scientific, Netherlands) and 250 mg of solid debris for DNA extraction.

DNA extraction

Bacterial DNA was extracted using the DNeasy® PowerSoil Pro Kit (Qiagen) following the manufacturer's protocol.[24] Specifically, we mixed 250 mg of the solid debris obtained as the fermentation end products with 800 µl of CD1 solution in 2 mL power bead pro tubes containing fine ceramic beads provided in the kit. We then mechanically lysed the samples using a vortex adapter fitted on an IKA® Vortex Genius 3 machine for 12 min. The unwanted sample matrix was removed by centrifugation at 15 000*g* for 1 min to collect the DNA-containing supernatant. We transferred the supernatant to new MB spin columns for a series of washing steps. We eluted DNA using 90 µl of elution buffer solution. We quantified and qualified the isolated DNA concentration using a NanoDrop™ ND-2000 and Qubit™ 4 fluorometer (Thermo Fisher Scientific, UK).

Sample preparation and analysis of short-chain fatty acids

The supernatant fraction from the fermentation end products was filtered using a 0.2 µm nylon filter to remove debris. 0.25 mL of cold Carrez A solution (0.1 M potassium ferrocyanide trihydrate) was added to 0.5 mL of filtered sample and mixed by vortexing. A further 0.25 mL of cold Carrez B solution (0.2 M zinc sulfate heptahydrate) was added and vortexed to precipitate the protein in the sample. The resulting solutions were centrifuged at 13 000*g* for 5 min to remove the precipitate. The precipitate-free aliquot was pipetted into clean HPLC standard vials (1.5 mL) for short-chain fatty acid quantification.

We quantified acetate, propionate, butyrate, isobutyrate, succinate, formate and lactate in the samples by High-Performance Liquid Chromatography (HPLC, Thermo Fisher Scientific, the Netherlands) according to Van Mastrigt *et al.*[25] Compounds were separated using an UltiMate 3000 HPLC (Dionex, Germany) equipped with an Aminex HPX-87H column (300 × 7.8 mm) with a guard column (Bio-Rad). As the mobile phase, 5 mM H_2SO_4 was used at a flow rate of 0.6 mL min^{-1}. The column was kept at a temperature of 40 °C. The compounds were quantified using a refractive index detector (RefractoMax520).

16S RNA sequencing and pre-analysis processing

To identify the bacterial communities in the DNA extracts, we performed sequencing of the V3–V4 hypervariable region of the 16S rRNA gene amplicon using the NovoSeq Illumina 6000 (strategy PE250) platform at Novogene Europe (Cambridge, UK). The polymerase chain reaction (PCR) was used to amplify the gene amplicons *via* 2% gel electrophoresis, using primers 341F (CCTAYGGGRBGCASCAG) and 806R

(GGACTACNNGGGTATCTAAT). Equal amounts of PCR products for each sample were pooled, end-repaired, A-tailed, and ligated with Illumina adaptors.

Paired-end reads (250 bp) were generated and subsequently merged using the fast length adjustment of short reads (FLASH) software.[26] The resulting paired-end fastq files were merged, denoised, and filtered to remove chimeric reads using the DADA2 open-source R package.[27] This yielded high-quality, amplicon-sequenced variants (ASVs) by retaining sequences with an abundance of 5% or higher. These ASVs obtained from this pipeline represent a cleaned dataset that enabled us to identify bacterial taxa and estimate their relative abundance in the samples. Using ASVs, metadata, and taxonomy, a phyloseq object was constructed using the phyloseq R package. Rarefaction was performed on a phyloseq object[28] using the rarefy_even_depth ( ) targeting the minimum read (see Fig. S1).

Statistical analysis

Variations in the gut microbiota upon exposure to treatments. The extracted DNA was analysed to determine the relative abundances of bacteria at the phyla and genus levels. Using an R-based package, the Fantaxtic R package[29] (version 2.0.1), we calculated relative abundances at the phylum and genus levels for the treatments and generated stacked bar plots of bacterial composition.[30] Principal Coordinate Analysis (PCoA) compared the similarity of the gut microbiota of the treatments based on the Bray–Curtis ecological distances.[31] Here, using PCoA ordination, we also compared the gut microbiota of consumers and non-consumers for the different treatments based on the analysis of similarities (ANOSIM) statistics.

To demonstrate the beta diversity of the gut microbiota from the different treatments, a pairwise Permutational Multivariate Analysis of Variance (PERMANOVA) model was used to compare dissimilarities between treatment groups, in particular, the vegan package[32] (version 2.6–4) using the function pairwise_adonis. For correction of multiple testing, the Bonferroni *post-hoc* test was used to adjust *p*-values in PERMANOVA.[33]

Discriminating genera in the gut microbiota upon exposure to treatments. For the identification of discriminant genera in the gut microbiota exposed to the treatments, a Linear Discriminant Effect Size (LEfSe) analysis was conducted based on a Linear Discriminant Analysis (LDA) score threshold of 4.0[34] with correction for multiple testing using a false discovery rate (FDR) of 5%.

Variations in the production of SCFAs upon exposure to treatments. Principal Component Analysis[35] (PCA) was used to ordinate the relative concentrations of SCFAs in the different treatments of consumers and non-consumers of Mabisi. Cohen's D effect sizes for each SCFA were calculated to assess pairwise differences between consumers and non-consumers of Mabisi. The formula indicated in eqn (1) was used to generate Cohen's D effect size values for each SCFA.[36]

$$\text{Effect size} = \frac{\text{mean SCFA conc. Mabisi} - \text{mean SCFA conc. controls}}{\text{pooled standard deviation}} \quad (1)$$

Here, mean SCFA concentrations from the stools of consumers/non-consumers and their respective negative controls were calculated and divided by their pooled standard deviation to generate an effect size. The larger the number, the greater the effect. Effect sizes on all SCFAs for consumers and non-consumers were calculated to compare the two (see Table S2).

Results

Shifts in Mabisi, FOS, and sterile water treated gut microbiota

Overall, the top three phyla in the gut microbiota exposed to Mabisi, FOS, and sterile water included Firmicutes, Actinobacteria, and Proteobacteria (Fig. 2). At the genus level, *Enterococcus*, *Sarcina*, *Streptococcus*, *Bifidobacterium*, and *Collinsella* were among the top five. Stools exposed to FOS resulted in a higher relative abundance of *Bifidobacterium* compared to sterile water, but this difference was only significant for FOS (*t*-test estimate = −24.18, $P_{adj} = 0.007$).

Type of stool (consumers and non-consumers of Mabisi) explained 3% of the total variation in the gut microbiota (PERMANOVA, Table 1; $p = 0.001$), with experimental treatment explaining 6% of the variation in the gut microbiota ($p = 0.001$) (Table 1). Mabisi treatment differed from sterile water- ($R^2 = 0.04$, *p*-adjusted = 0.003) and FOS-treated gut microbiota (*p*-adjusted = 0.003). Pairwise PERMANOVA revealed shifts in the Mabisi-treated gut microbiota of non-consumers that were statistically higher ($R^2 = 0.08$, $P_{adj} = 0.015$) compared to the Mabisi-treated gut microbiota of consumers ($R^2 = 0.07$, $P_{adj} = 0.375$) (see Table S1). Beta diversity analysis revealed significant differences among the six donors ($R^2 = 0.68$, $p = 0.001$), with Mabisi consumers clustering together under treatment compared to non-consumers (see Fig. S2 and S3).

Effects on the gut microbiota treated with Mabisi, FOS, and sterile water were further analysed for the similarities using PCoA. The analysis of similarities (AnoSim) test yielded a global *R* statistic of 0.092, indicating a moderate level of dissimilarity among the treated gut microbiota groups (Fig. 3). The *p*-value of 0.001 indicates that there are statistically significant differences among the treatment groups and stool donors. Inspecting the PCoA suggests the first axis to be associated with the treatment, and the second axis – more clearly – seems associated with the stool donor category (Mabisi consumer or Mabisi non-consumer). We observe three distinct outliers (see Fig. 3, separating along axis 2), which are technical replicates of stool treated with FOS from one distinct child who is a non-consumer of Mabisi.

Discriminating genera in the gut microbiota treated with Mabisi, FOS, and sterile water

We employed LEfSe with a stringent LDA score threshold of 4 to discern differentiating genera in the gut microbiota from Mabisi consumers and non-consumers treated with Mabisi, FOS, and sterile water. Notably, the Mabisi-treated gut microbiota showed strong associations with *Pediococcus*. Fructooligosaccharide (FOS)-treated gut microbiota were

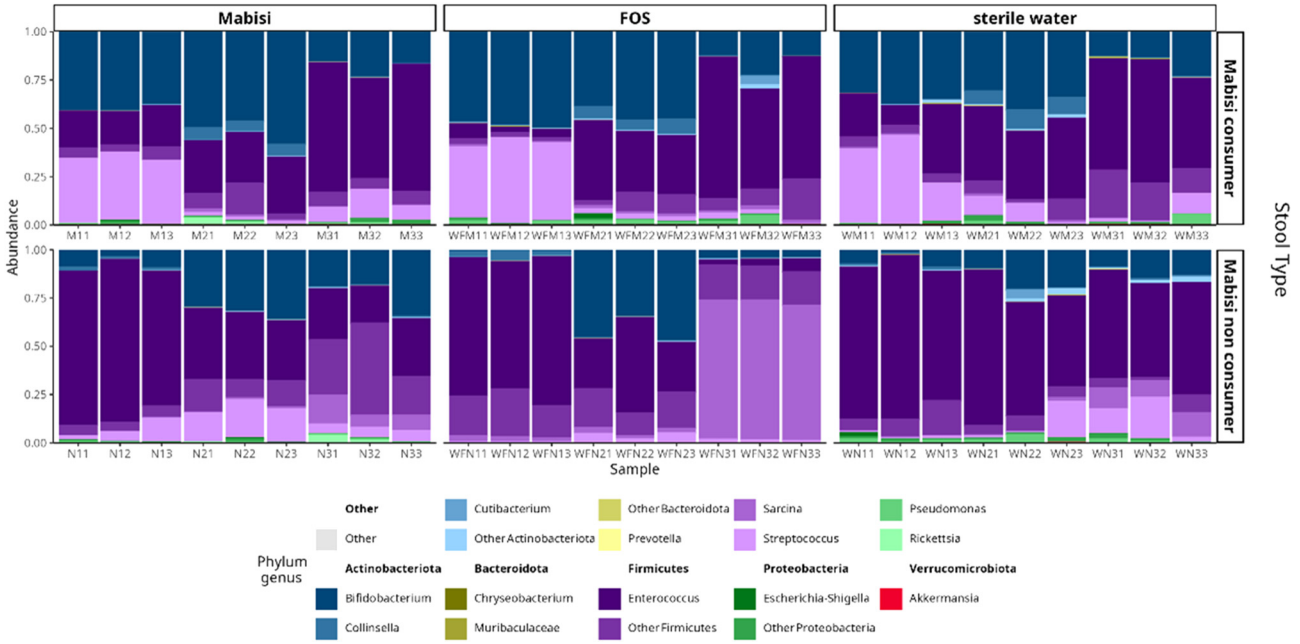

Fig. 2 Nested bar charts showing the relative abundance of the bacterial composition at the phyla and genus levels for Mabisi, FOS, and sterile water of consumers and non-consumers of Mabisi. Note: sterile water is the negative control.

Table 1 Analysis of the gut microbiota composition using PERMANOVA, evaluating the effects of stool type, treatment, and their interaction

Variables	D_f	Sums of squares	R^2	*F*-model	*P*-value	*P*-adjusted
Type of stool (consumers and non-consumers)	1	0.72	0.03	1.59	0.001	0.001*
Treatment groups (Mabisi, FOS and sterile water)	2	1.51	0.06	1.68	0.001	0.001*
Interaction						
Type of stool × treatment	2	1.21	0.01	1.38	0.001*	
Treatment group pairwise comparison						
Mabisi *vs.* sterile water	1	0.73	0.04	1.57	0.001	0.003*
Mabisi *vs.* FOS	1	0.82	0.05	1.79	0.001	0.003*
Sterile water *vs.* FOS	1	0.72	0.04	1.55	0.002	0.006*

Pairwise comparisons with adjusted *p*-values are presented. Asterisks (*) indicate statistically significant differences, while the multiplication sign (×) represents interaction effects. Note: sterile water is the negative control.

strongly linked to *Sarcina*. The sterile water-treated gut microbiota was characterized by weaker associations with *Staphylococcus* and *Paraclostridium*; however, they were not statistically significant at an LDA score of 4 (see Fig. 4A). We observed similar discriminating genera outcomes in both Mabisi-treated gut microbiota of consumers and non-consumers (see Fig. 4B).

Production of SCFAs

SCFA production in Mabisi, FOS, and sterile water treated gut microbiota was analysed and visualized using Principal Component Analysis (PCA). Among the treatments, the first two principal components (PC1 and PC2) accounted for 63.9% of the total variation in the production of SCFAs. The production of acetate, butyrate, propionate, isobutyrate, succinate, lactate, and formate clustered closer to Mabisi, FOS and sterile water (Fig. 5A). Furthermore, we found subtle differences in SCFA production between consumers and non-consumers, with non-consumers producing relatively more acetate, butyrate, formate and succinate (see Cohen's D scores in Table S2).

We further compared SCFA production in the treatments using box plots (Fig. 5B, also for statistical details, see Fig. S4–S6). We found lactate to be significantly higher (F_{Welch} = 8.6, $P_{\text{fdr adj}}$ = 0.003) in the Mabisi-treated gut microbiota compared to those treated with sterile water and FOS (see Fig. S4).

Discussion

We assessed the effect of Mabisi exposure on gut microbiota composition and SCFA production using stool from Mabisi consumers and non-consumers. The top phyla of the gut microbiota of both consumers and non-consumers are *Firmicutes*, *Actinobacteria*, and *Proteobacteria*. This composition at the phylum level represents the typical composition of the

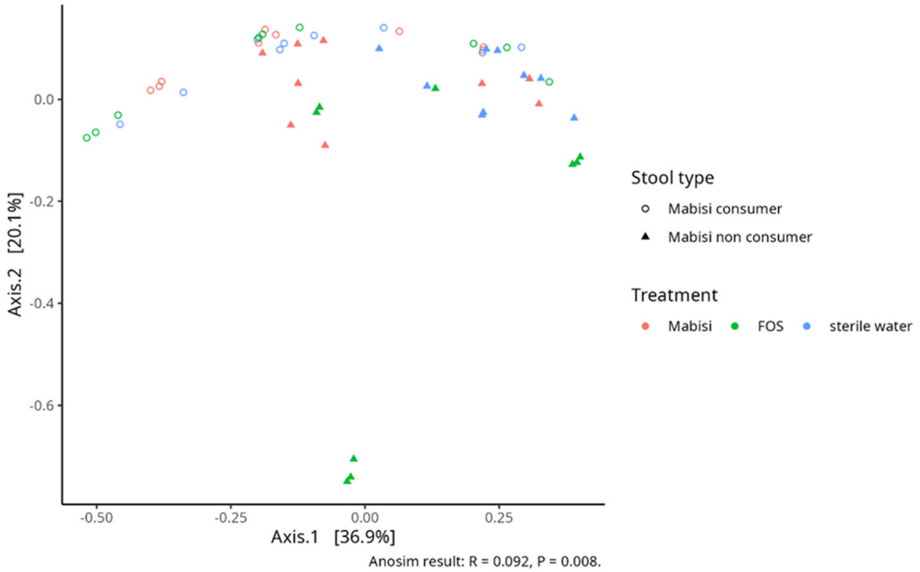

Fig. 3 Principal coordinate analysis (PCoA) of the gut microbiota treated with Mabisi, FOS and sterile water exposed to stool of consumers and non-consumers of Mabisi for axis 1 *vs.* 2.

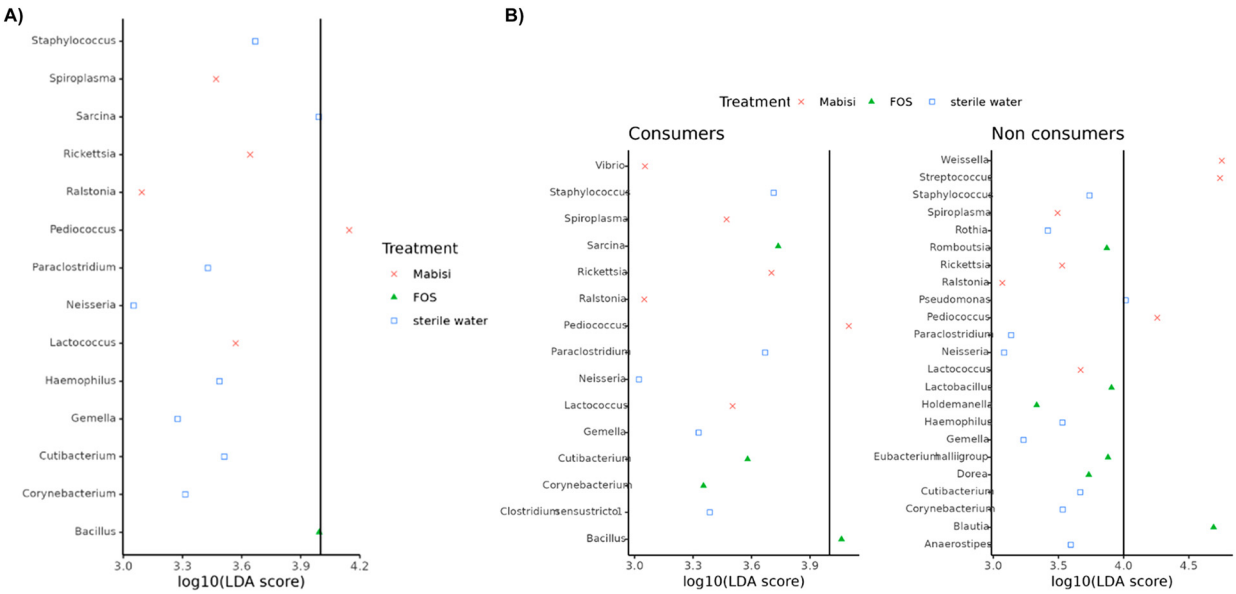

Fig. 4 (A) Linear discriminant effect size analysis (LEfSe) of the gut microbiota treatment groups analysed for both consumers and non-consumers using an LDA score cut-off of 4 and an FDR of 5%. (B) LEfSe analysis of discriminant genera as shown for the gut microbiota treatment of Mabisi consumers' and non-consumers' stools.

human gut microbiota, indicating a successful experiment setup and execution. The top phyla all harbour both strict and facultative anaerobic bacteria that are inherent to the human colon.

Mabisi exposure showed modulation towards an increased abundance of *Pediococcus*. Bacteria from this genus are known to have beneficial effects on humans.[37–40] Specifically, *Pediococcus* is a genus of Gram-positive, homofermentative bacteria mainly producing lactic acid. They are known at the strain level to protect the human gut by the production of pediocin, metabolites which may suppress colonization by pathogenic species, for instance, *Listeria*. Furthermore, pediocin-producing species, including *Pediococcus pentosaceus* and *Pediococcus acidilactici*, are applied in the food industry for pediocin production. Pediocin is added to various fermentation processes to prevent contamination by pathogenic species.[39] Recently, in therapeutics, *Pediococcus* has been used as a probiotic supplement to support the gut health of

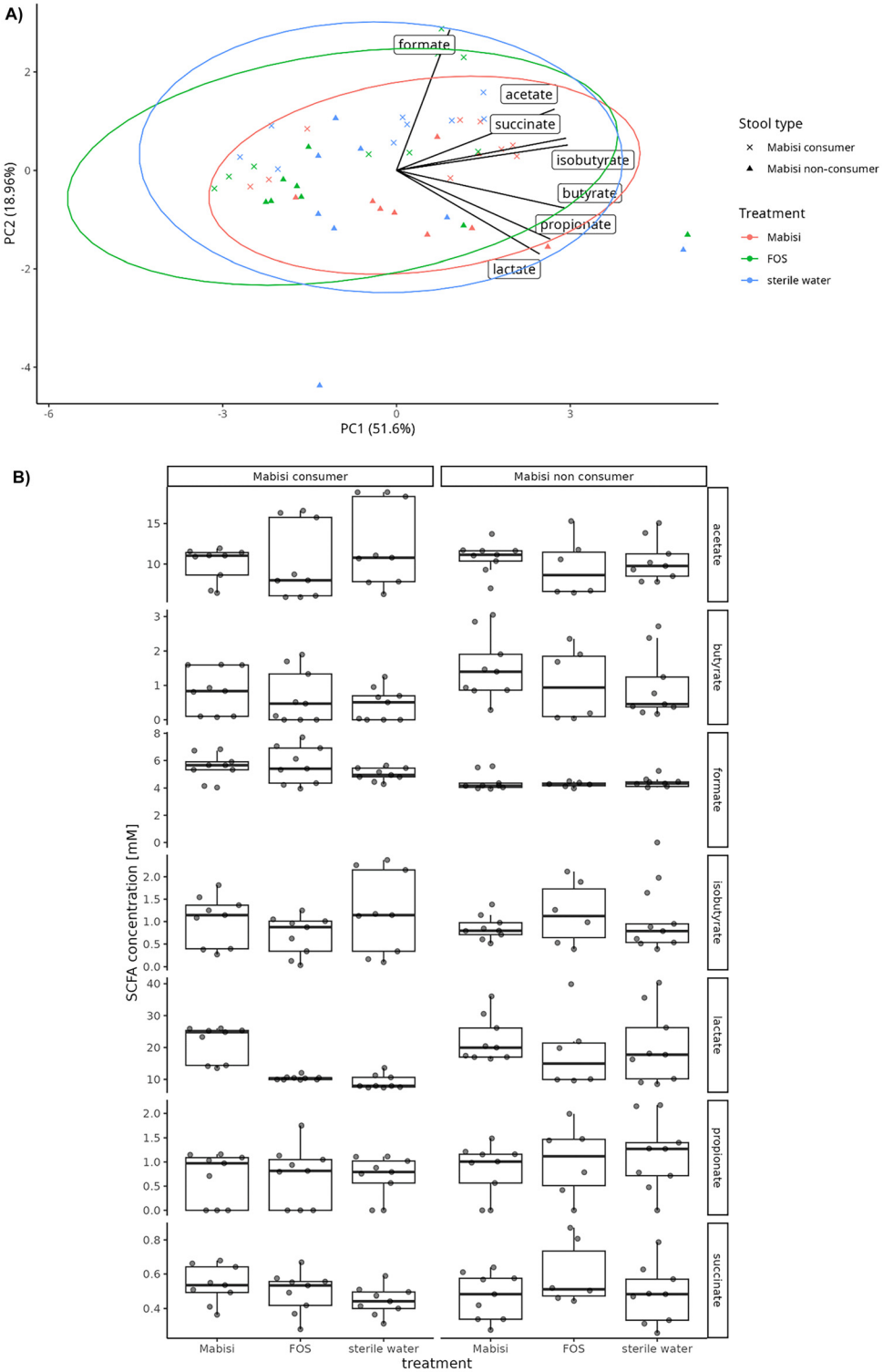

Fig. 5 (A) Principal component analysis of SCFA production in the treated gut microbiota from consumers and non-consumers of Mabisi. (B) Box plot showing the actual SCFA concentration in the treated gut microbiota from consumers and non-consumers of Mabisi. Note: sterile water is the negative control. Also check Fig. S4–S6 for statistical details.

animals and humans.[38] Some *Pediococcus* strains survive human digestion despite its harsh environment characterized by low pH and the presence of bile acids. Mabisi may have limited counts of *Pediococcus* compared to commercially prepared probiotic products. However, with habitual consumption, *Pediococcus* strains may impact hosts' guts by the pro-

duction of SCFAs from the metabolization of oligosaccharides. Most TFFs are believed to have probiotic or prebiotic effects on the human digestive system.[16,41–44] Demonstrating the mode of action of TFFs on human gut health creates opportunities for their commercialization and use in the therapeutic industry. Furthermore, by identifying and isolating wild-type bacterial strains in TFFs with efficacy to modulate the human gut microbiota, novel products enriched with specific bacteria from TFFs could be developed for specific populations. Additionally, this promotes their use as natural indigenous products with known benefits for use by a wider population.

Both treatment (Mabisi, FOS and sterile water) and the type of stool (Mabisi consumer or non-consumer) significantly impacted the gut microbiota composition (PERMANOVA, Table 1). Importantly, the interaction between treatment and the type of stool was significant, suggesting that the effect of Mabisi exposure is different for the different types of stool. Indeed, non-consumers of Mabisi had statistically greater shifts in the gut microbiota composition following Mabisi exposure compared to consumers, as indicated by a higher fraction of explained variation shown for non-consumers than for consumers in the PERMANOVA. This finding is further supported by pairwise PERMANOVA analysis, which shows that the gut microbiota from the Mabisi-treated samples of consumers exhibit a significantly greater similarity to their negative controls compared to those of non-consumers. Hence, we deduce that the gut microbiota of consumers is already pre-conditioned to Mabisi exposure compared to non-consumers based on limited shifts realized after treatment with Mabisi. This ecological stability analogy is supported by a review by Lozupone *et al.* that postulates that an increase in shifts in the gut microbiota may indicate instability, more especially after exposure to external pressures that could be pathogens, deleterious metabolites, exposure to antibiotics or simply a change in nutrients as seen in non-consumers.[45] For instance, the gut microbiota composition in patients undergoing dysbiosis (imbalance of the gut microbiota composition) related to a specific pathology may drastically shift, indicating an increase in gut microbiota variations that could be restored to its original state by using prebiotics and probiotics.[46] We acknowledge that this ecological analogy has its limitations in that it fails to account for the direction of shift and intricate details of change.

In this experiment, we found a high level of production of SCFAs in non-consumers than in consumers. Taylor and colleagues studied the effect of consumption of fermented foods in consumers *versus* non-consumers involving 7000 participants.[47] Subtle, yet statistically significant, differences in the beta diversity of the two groups were revealed in their gut metabolome, which showed that consumers of plant-based fermented foods had a higher production of conjugated linoleic acid compared to non-consumers. For non-consumers of Mabisi in this study, specifically acetate, butyrate, formate, and succinate increased. Our interpretation is that Mabisi, as a new food for non-consumers, could have been an advantage since their gut may be used to extract SCFAs from other products such as proteins and fibres. The higher production of SCFAs in non-consumers demonstrates a possible use of Mabisi as a base preparation product of choice for therapeutic food that improves the health of humans, especially children with active intestinal pathologies. Mabisi may have a similar effect as demonstrated in a randomized controlled trial involving Kefir, another dairy-based food.[48] Kefir modulated the gut microbiota of individuals with ulcerative colitis and Crohn's disease by increasing the abundance of lactic acid-producing bacteria, thereby improving the patient's quality of life.

Fructooligosaccharide (FOS)-treated gut microbiota were mainly linked to *Sarcina*, *Bacillus*, and *Clostridium*_sensu_stricto_1, also suggesting distinctive microbial signatures associated with this treatment. Overall, FOS treatment resulted in a higher relative abundance of *Bifidobacterium* than Mabisi treatments. *Bifidobacterium* species are further known for their ability to produce short-chain fatty acids and vitamins, which support the overall immune function and promote the integrity of the gut barrier.[49] Their presence in sufficient quantities is associated with improved digestion, reduced inflammation, and enhanced nutrient absorption, highlighting their significance in fostering a healthy gut environment and overall well-being.

A limitation of this experiment is that the model system used is simple and does not explicitly mimic the physiology of normal human digestion. Targeting specific beneficial species in stool exposed to Mabisi using specific primers could have increased the resolution of differences between consumers and non-consumers. A key limitation of this study is the inherent intra- and inter-individual variability in the gut microbiota composition, which complicates the interpretation of changes following Mabisi treatment. The differences observed in microbial responses may be influenced by each individual's baseline gut microbiota (see Fig. S3). Furthermore, employing, for instance, nanopore technology to identify bacterial species based on long 16S amplicon reads may provide more insights into specific bacteria in treatments. On the other hand, the design of this study was robust as it informed variations in the gut microbiota and SCFA production using minimal resources. Furthermore, this study paves the way for further study of Mabisi's probiotic and prebiotic effects on the gut microbiota using experimental models.

In conclusion, we assessed the effect of Mabisi on the gut microbiota and SCFA production using stool material from Mabisi consumers and non-consumers. Our findings revealed subtle but important shifts in the gut microbiota of non-consumers compared to that of Mabisi consumers. Notably, non-consumers had a higher level of SCFA production compared to Mabisi consumers, with increased concentrations of acetate, butyrate, formate, and succinate observed in the former. Revealing the effects of Mabisi based on shifts in the Mabisi-treated gut microbiota is an important aspect in promoting the consumption of most under-studied TFFs with potential beneficial effects in humans. Mabisi exposure modulates the gut microbiota by promoting beneficial bacteria and the production of SCFAs. Mabisi could be suitable for the preparation of therapeutic food for the management of digestive disorders

that may need an influx of SCFAs. Prominent to this study is the demonstration of the stability of Mabisi consumers' gut microbiota compared to non-consumers. Further studies to determine the nature of the effect of Mabisi on the gut microbiota are needed to establish its possible future use. Such studies could focus on specific components of Mabisi for their effects on the gut microbiota and functionality. This study contributes to a strategy to investigate the most under-studied TFFs in low-income settings.

Author contributions

MN: conceptualization, methodology, data curation, formal analysis, visualization, project administration, writing – original draft, writing – review and editing, and funding acquisition. OM: data curation, formal analysis, visualization and validation. SB: data curation, formal analysis and visualization. JS: conceptualization, project administration, writing – original draft, writing – review and editing, validation and supervision. ET: conceptualization, project administration, writing – original draft, validation, writing – review and editing, supervision and funding acquisition. BZ: conceptualization, methodology, data curation, formal analysis, visualization, project administration, validation, writing – original draft, writing – review and editing, supervision and funding acquisition. SS: conceptualization, methodology, data curation, formal analysis, visualization, project administration, validation, writing – original draft, writing – review and editing, supervision and funding acquisition.

Conflicts of interest

The authors declare no conflict of interest.

Data availability

Metadata and R scripts have been deposited in the GitHub repository at **https://git.wur.nl/mabisi-research**. Raw 16S sequences have been deposited in the Zenodo repository at **https://zenodo.org/uploads/10259966**.

Supplementary information is available. The supplemenatry material comprise of information on rarefaction of samples species sequences, top abundant taxa in both gut microbiota of consumers and non-consumers treated with Mabisi, beta diversity analysis and SCFA production. See DOI: **https://doi.org/10.1039/d5fo01627d**.

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References

1 H. B. Moonga, S. E. Schoustra, J. van den Heuvel, A. R. Linnemann, M. S. Samad, J. Shindanoo, *et al.* Composition and Diversity of Natural Bacterial Communities in Mabisi, a Traditionally Fermented Milk, *Front. Microbiol.*, 2020, **11**, 1816.

2 M. Jaagura, N. Part, K. Adamberg, J. Kazantseva and E. Viiard, Consumption of multi-fiber enriched yogurt is associated with increase of Bifidobacterium animalis and butyrate producing bacteria in human fecal microbiota, *J. Funct. Foods*, 2022, **88**, 104899.

3 K. Bauer-Estrada, C. Sandoval-Cuellar, Y. Rojas-Muñoz and M. X. Quintanilla-Carvajal, The modulatory effect of encapsulated bioactives and probiotics on gut microbiota: improving health status through functional food, *Food Funct.*, 2023, **14**(1), 32–55.

4 S. O. Salehi, F. Karimpour, H. Imani, M. A. Ghatee, M. Pirouze and T. Bahramfard, Effects of an Iranian traditional fermented food consumption on blood glucose, blood pressure, and lipid profile in type 2 diabetes: a randomized controlled clinical trial, *Eur. J. Nutr.*, 2022, **61**(7), 3367–3375.

5 S. M. Devi, N. K. Kurrey and P. M. Halami, In vitro anti-inflammatory activity among probiotic Lactobacillus species isolated from fermented foods, *J. Funct. Foods*, 2018, **47**, 19–27.

6 T. Kanai, K. Matsuoka, M. Naganuma, A. Hayashi and T. Hisamatsu, Diet, microbiota, and inflammatory bowel disease: lessons from Japanese foods, *Korean J. Intern. Med.*, 2014, **29**(4), 409–415.

7 L. Kairey, B. Leech, F. El-Assaad, A. Bugarcic, D. Dawson and R. Lauche, The effects of kefir consumption on human health: a systematic review of randomized controlled trials, *Nutr. Rev.*, 2023, **81**(3), 267–286.

8 C. Folke, S. Carpenter, B. Walker, M. Scheffer, T. Elmqvist, L. Gunderson, *et al.*, Regime Shifts, Resilience, and Biodiversity in Ecosystem Management, *Annu. Rev. Ecol. Evol. Syst.*, 2004, **35**(1), 557–581.

9 H. B. Moonga, S. E. Schoustra, A. R. Linnemann, E. Kuntashula, J. Shindano and E. J. Smid, The art of mabisi production: A traditional fermented milk, *PLoS One*, 2019, **14**(3), e0213541.

10 S. E. Schoustra, C. Kasase, C. Toarta, R. Kassen and A. J. Poulain, Microbial community structure of three traditional zambian fermented products: mabisi, chibwantu and munkoyo, *PLoS One*, 2013, **8**(5), e63948.

11 B. T. Hamaimbo, P. A. Marinda, V. Nyau, J. Chileshe, C. Khayeka-Wandabwa and S. E. Schoustra, Adequate Dietary Intake and Consumption of Indigenous Fermented Products Are Associated with Improved Nutrition Status

among Children Aged 6–23 Months in Zambia, *Dairy*, 2023, **4**(1), 137–149.

12 J. Chileshe, J. Sikalima, P. Malunga, R. Handema, B. Zwaan, E. Talsma, *et al.*, Do traditional fermented foods affect the gut microbiota metabolism? Assessing the potential using an in vitro system, *academia.edu*, 2019, **72**, DOI: **10.1039/C3FO60702J**.

13 A. Y. Alekseeva, A. E. Groenenboom, E. J. Smid and S. E. Schoustra, Eco-Evolutionary Dynamics in Microbial Communities from Spontaneous Fermented Foods, *Int. J. Environ. Res. Public Health*, 2021, **18**(19), 10093.

14 A. I. Alekseeva, K. Ye, J. Mentani, J. C. M. Wolkers-Rooijackers, E. J. Smid and S. E. Schoustra, Traditional fermented milk product from Zambia shifts the gut microbiota to healthier metabolism in a simulated SHIME® model system. 2025 Mar 31 [cited 2025 May 22]; Available from: **https://brill.com/view/journals/bm/aop/article-10.1163-18762891-bja00068/article-10.1163-18762891-bja00068.xml**.

15 J. Chileshe, Nutrition, health and microbial ecology of traditional fermented foods in Zambia. 2019 [cited 2024 Apr 18]; Available from: **https://research.wur.nl/en/publications/nutrition-health-and-microbial-ecology-of-traditional-fermented-f**.

16 S. Pérez-Burillo, T. Mehta, S. Pastoriza, D. L. Kramer, O. Paliy and J. Á. Rufián-Henares, Potential probiotic salami with dietary fiber modulates antioxidant capacity, short chain fatty acid production and gut microbiota community structure, *LWT*, 2019, **105**, 355–362.

17 M. Minekus, M. Alminger, P. Alvito, S. Ballance, T. Bohn, C. Bourlieu, *et al.*, A standardised static in vitro digestion method suitable for food–an international consensus, *Food Funct.*, 2014, **5**(6), 1113–1124.

18 A. Brodkorb, L. Egger, M. Alminger, P. Alvito, R. Assunção, S. Ballance, *et al.*, INFOGEST static in vitro simulation of gastrointestinal food digestion, *Nat. Protoc.*, 2019, **14**(4), 991–1014.

19 S. Pérez-Burillo, S. Molino, B. Navajas-Porras, Á. J. Valverde-Moya, D. Hinojosa-Nogueira, A. López-Maldonado, *et al.*, An in vitro batch fermentation protocol for studying the contribution of food to gut microbiota composition and functionality, *Nat. Protoc.*, 2021, **16**(7), 3186–3209.

20 T. Van de Wiele, P. Van den Abbeele, W. Ossieur, S. Possemiers and M. Marzorati, The simulator of the human intestinal microbial ecosystem (SHIME®), in *Impact Food Bioact Health Vitro Ex Vivo Models*, 2015, pp. 305–317.

21 M Minekus, The TNO gastro-intestinal model (TIM), *The Impact of Food Bioactives on Health: in vitro and ex vivo models*, 2015, pp. 37–46.

22 S. Kang, J. Niu, Q. Zhang, X. Zhang, G. Han and M. Zhao, Niche differentiation is the underlying mechanism maintaining the relationship between community diversity and stability under grazing pressure, *Glob. Ecol. Conserv*, 2020, **24**, e01246.

23 M. Nyati, S. Schoustra, J. Shindano, B. J. Zwaan and E. F. Talsma , Exploring the gut microbiota of young children in Zambia: identifying potential determinants for a low-income environment.

24 . Powersoilkit – Google Search. [cited 2024 Apr 16]. Available from: **https://www.google.com/search?q=Powersoilkit&rlz=1C1GCEB_enNL1063NL1063&oq=Powersoilkit&gs_lcrp=EgZjaHJvbWUyBggAEEUYOTIJCAEQABgNGIAEMgkIAhAAGA0YgAQyCAgDEAAYDRgeMgoIBBAAGAgYDRgeMgoIBRAAGIAEGKIEMgoIBhAAGIAEGKIEMgoIBxAAGIAEGKIE0gEKMTA3NThqMGoxNagCCLACAQ&sourceid=chrome&ie=UTF-8**.

25 O. van Mastrigt, E. E. Mager, C. Jamin, T. Abee and E. J. Smid, Citrate, low pH and amino acid limitation induce citrate utilization in Lactococcus lactis biovar diacetylactis, *Microb. Biotechnol.*, 2018, **11**(2), 369–380.

26 T. Magoč and S. L. Salzberg, FLASH: fast length adjustment of short reads to improve genome assemblies, *Bioinformatics*, 2011, **27**(21), 2957–2963.

27 B. J. Callahan, P. J. McMurdie, M. J. Rosen, A. W. Han, A. J. A. Johnson and S. P. Holmes, DADA2: High-resolution sample inference from Illumina amplicon data, *Nat. Methods*, 2016, **13**(7), 581–583.

28 P. J. McMurdie and S. Holmes, phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data, *PLOS one*, 2013, **2**(7), 4–8.

29 G. M. Teunisse, *Fantaxtic – Nested Bar Plots for Phyloseq Data*. 2022. Available from: **https://github.com/gmteunisse/Fantaxtic** [cited 2024 Feb 9].

30 G. M. Teunisse, *Fantaxtic-Nested Bar Plots for Phyloseq Data*, 2022.

31 K. R. Clarke, P. J. Somerfield and M. G. Chapman, On resemblance measures for ecological studies, including taxonomic dissimilarities and a zero-adjusted Bray–Curtis coefficient for denuded assemblages, *J. Exp. Mar. Biol. Ecol.*, 2006, **330**(1), 55–80.

32 J. Oksanen, F. G. Blanchet, R. Kindt, P. Legendre, P. R. Minchin, R. B. O'hara, *et al.*, Package 'vegan', Community Ecol Package Version, *Community Ecology*, 2013, **2**(9), 1–295.

33 D. Perrett, J. Schaffer, A. Piccone and M. Roozeboom, Bonferroni adjustments in tests for regression coefficients, *Mult. Linear Reg. Viewpoints*, 2006, **32**(1), 1–6.

34 F. Chang, S. He and C. Dang, Assisted Selection of Biomarkers by Linear Discriminant Analysis Effect Size (LEfSe) in Microbiome Data, *J. Visualized Exp.*, 2022, **16**, e61715.

35 I. Irnawati, F. D. O. Riswanto, S. Riyanto, S. Martono and A. Rohman, The use of software packages of R factoextra and FactoMineR and their application in principal component analysis for authentication of oils, *Indones. J. Chemom. Pharm. Anal.*, 2021, 1–10.

36 R. Rosenthal, H. Cooper and L. Hedges, Parametric measures of effect size, *Handb. Res. Synth.*, 1994, **621**(2), 231–244.

37 J. M. W. Wong, R. de Souza, C. W. C. Kendall, A. Emam and D. J. A. Jenkins, Colonic Health: Fermentation and Short Chain Fatty Acids, *J. Clin. Gastroenterol.*, 2006, **40**(3), 235–243.

38 S. Jiang, L. Cai, L. Lv and L. Li, Pediococcus pentosaceus, a future additive or probiotic candidate, *Microb. Cell Fact.*, 2021, **20**(1), 45.

39 M. C. W. Porto, T. M. Kuniyoshi, P. O. S. Azevedo, M. Vitolo and R. P. S. Oliveira, *Pediococcus* spp.: An important genus of lactic acid bacteria and pediocin producers, *Biotechnol. Adv.*, 2017, **35**(3), 361–374.

40 M. d. Carmo, C. i. d. Santos, M. C. Araújo, J. A. Girón, E. S. Fernandes and V. Monteiro-Neto, Probiotics, mechanisms of action, and clinical perspectives for diarrhea management in children, *Food Funct.*, 2018, **9**(10), 5074–5095.

41 S. Pérez-Burillo, T. Mehta, A. Esteban-Muñoz, S. Pastoriza and O. Paliy, Ángel Rufián-Henares J. Effect of in vitro digestion-fermentation on green and roasted coffee bioactivity: The role of the gut microbiota, *Food Chem.*, 2019, **279**, 252–259.

42 F. A. Tomás-Barberán and J. C. Espín, Effect of food structure and processing on (Poly) phenol–gut microbiota interactions and the effects on human health, *Annu. Rev. Food Sci. Technol.*, 2019, **10**, 221–238.

43 W. Liao, W. Li, S. Liu, D. Tang, Y. Chen, Y. Wang, *et al.*, Potential prebiotic effects of nonabsorptive components of Keemun and Dianhong black tea: an in vitro study, *Food Sci. Hum. Wellness*, 2022, **11**(3), 648–659.

44 L. Pozzo, C. Alcántara, M. Selma-Royo, I. Garcia-Mantrana, E. Bramanti, V. Longo, *et al.*, The impact of sourdough fermentation of spelt (Triticum dicoccum) from Garfagnana on gut microbiota composition and in vitro activity, *J. Funct. Foods*, 2022, **91**, 105007.

45 C. A. Lozupone, J. I. Stombaugh, J. I. Gordon, J. K. Jansson and R. Knight, Diversity, stability and resilience of the human gut microbiota, *Nature*, 2012, **489**(7415), 220–230.

46 Y. L. Tsai, T. L. Lin, C. J. Chang, T. R. Wu, W. F. Lai, C. C. Lu, *et al.*, Probiotics, prebiotics and amelioration of diseases, *J. Biomed. Sci.*, 2019, **26**(1), 3.

47 B. C. Taylor, F. Lejzerowicz, M. Poirel, J. P. Shaffer, L. Jiang, A. Aksenov, *et al.*, Consumption of Fermented Foods Is Associated with Systematic Differences in the Gut Microbiome and Metabolome, *mSystems*, 2020, **5**(2), e00901–e00919.

48 İ. Yılmaz, M. E. Dolar and H. Özpınar, Effect of administering kefir on the changes in fecal microbiota and symptoms of inflammatory bowel disease: A randomized controlled trial, *Turk. J. Gastroenterol.*, 2019, **30**(3), 242–253.

49 S. A. Joyce and C. G. M. Gahan, The gut microbiota and the metabolic health of the host, *Curr. Opin. Gastroenterol.*, 2014, **30**(2), 120–127.