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Glycosidic linkage structures influence dietary fiber fermentability and propionate production by human colonic microbiota *in vitro*

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Abbreviations: CUL, KUHIMM without fiber addition; **DEX-1**, indigestible dextrin-1; **DEX-2**, indigestible dextrin-2; **DEX-3**, indigestible dextrin-3; **FEC**, fecal inoculum; **GAM**, Gifu anaerobic medium; **HPLC**, high-performance liquid chromatography; **KUHIMM**, Kobe University Human Intestinal Microbiota Model; **IMD,** isomaltodextrin; **NGS,** next-

generation sequencing; **OTU**, operational taxonomic unit; **PDX,** polydextrose; **RGN,** resistant glucan; **SCFA,** short-chain fatty acid;

Abstract

Some dietary fibers can be produced by starch modification; however, information regarding the relationships between glycosidic linkages and dietary fiber fermentability or the production of short-chain fatty acids is limited. Thus, we investigated these relationships using an *in vitro* model of human colonic microbiota, which approximates the bacterial species richness and diversity in inoculated fecal samples. Six dietary fibers with various glycosidic linkage contents were prepared. Each dietary fiber (final concentration: 1.0% by volume) was administered *in vitro* to human microbiota models 18 h after fecal samples were inoculated. The contents of (1 → 2) plus (1 → 3) linkages and β-linkages in the six dietary fibers negatively correlated with the fermentation speed and fermentation ratio of the indigestible parts of the dietary fibers (R^2 = 0.8126 or 0.8306 and R^2 = 0.9106 or 0.9673, respectively) 24 h after administering each dietary fiber. Further, the concentrations of propionate produced *in vitro* by human microbiota positively correlated with the fermentation speed and fermentation ratio (R^2 = 0.9149 and 0.9581, respectively). Our *in vitro* assay revealed that (1 → 2) plus (1 → 3) linkages and β-linkages in dietary fiber affect resistance to fermentation and propionate production by the human colonic microbiota.

1 Introduction

Dietary fiber is defined as a group of indigestible carbohydrate polymers naturally contained in food products and isolated or synthesized for addition to foods. Most countries have accepted the inclusion of polymers with three or more monomeric units as dietary fiber.[1] Dietary fiber is resistant to hydrolysis by digestive enzymes in the human small intestine, although it can be fermented in the caecum and colon and finally absorbed by the host.[2] Dietary fiber improves human health via microbiota-independent and -dependent manners by promoting beneficial microbes; limiting the growth, adhesion, and cytotoxicity of pathogenic microbes; and stimulating fiber-derived microbial short-chain fatty acid (SCFA) production.[3] Here, we focused on the microbiota-dependent effects of dietary fiber.

The human microbiota is constituted by numerous microbial communities comprising hundreds of individual microbial species. Glycosidic linkages in dietary fiber are broken down by a suite of enzymes, such as polysaccharidases and glycosidases.[4] Intriguingly, human enzymes are unable to digest most types of fiber molecules.[4] Thus, indigestible carbohydrates, especially soluble dietary fibers, are the primary energy source for most gut microbes, and can directly impact those species that heavily depend on such substrates.[5] Some gut bacteria possess enzymes to degrade complex polysaccharides into smaller oligomers and respective sugar components. Bacteria then ferment these smaller components into SCFAs, H_2, CO_2, and other end-products, which impacts many facets of host metabolism.[6] The physicochemical characteristics of dietary fiber influence bacterial digestion. For example, dietary fibers with simple chemical structures, such as fructooligosaccharides, require few bacterial glycoside hydrolases for degradation. In contrast, the digestion of more complex molecules, such as xyloglucans (which contain a range of sugar and linkage types), requires that bacteria have more glycoside hydrolases.[7] Thus, a comprehensive understanding of the relationships

between chemical structures and the fermentability of dietary fiber remains a matter of investigation.

Previously, we developed a model culture system for simulating the human colonic microbiota *in vitro* (named the Kobe University Human Intestinal Microbiota Model [KUHIMM]), which involves inoculating human fecal samples and retaining the bacterial species richness in the feces.[8,9] This model was validated by next-generation sequencing (NGS) analysis. Since the diversity of the bacterial proportions in fecal samples was retained, the KUHIMM enabled detection of decreased butyrogenesis in the microbiota of patients with ulcerative colitis,[9] and evaluation of the effects of prebiotic oligosaccharides, dietary fibers, and the probiotic strain, *Bacillus coagulans*.[8,10] Hence, it was worth improving the KUHIMM to evaluate the effects of the chemical structures of dietary fibers on the fermentability by the human colonic microbiota.

A novel type of soluble dietary fiber, exhibiting prebiotic properties, has been derived by modifying starch or glucose.[11,12] Glycosidic linkages other than α-(1 → 4), and α-(1 → 6) are formed during modification.[13] In this study, we evaluated the effect of linkage types in dietary fiber on their fermentability by human colonic microbiota, using the KUHIMM. We prepared various dietary fibers containing mixed linkages, including α or β-(1 → 4), (1 → 6), (1 → 2), and (1 → 3). Each dietary fiber was supplemented into the model culture system after the KUHIMM was constructed. We identified relationships between the linkage types in dietary fibers and their fermentability by the human colonic microbiota.

2 Materials and methods

2.1 Preparation of dietary fibers

The following commercial dietary fibers were prepared: resistant glucan (RGN, Fit Fiber #80; Nihon Shokuhin Kako, Tokyo, Japan), polydextrose (PDX, Litesse Ⅱ ; Dupont Nutrition

& Biosciences, Copenhagen, Denmark), indigestible dextrin-1 (DEX-1, NUTRIOSE FM06; Roquette, Lestrem, France), indigestible dextrin-2 (DEX-2, PROMITOR85; Tate & Lyle, London, England, UK), indigestible dextrin-3 (DEX-3, Fibersol-2; Matsutani Chemical Industry, Hyogo, Japan), and isomaltodextrin (IMD, Fibryxa; Hayashibara, Okayama, Japan). Methylation analysis was performed to determine the glycosidic linkage patterns of dietary fibers, according to the method developed by Cuican and Kerek.[14] Gas chromatography-mass spectrometry was performed using a BD-225 capillary column (30 m length × 0.25 mm inside diameter × 0.15 μm film; Agilent Technology, Santa Clara, CA, USA), which was heated to 170°C for 1 min, then heated to 210°C at a rate of 3 °C/min, and maintained at 210°C for 20 min. The injection temperature was 210°C, and the eluent was helium. The molecular weight was determined based on the average molecular weight number via high-performance liquid chromatography (HPLC) (Shimadzu Corporation, Kyoto, Japan).[15,16] The conditions for performing HPLC were as follows; column used: TSKgel G6000PWXL, G3000PWXL, G2500PWXL (300 mm × 7.8 mm inside diameter) (Tosoh, Tokyo, Japan); column temperature: 80 °C; elution: distilled water; flow rate: 0.5 mL min^{-1}; detector: RID-20A refractive-index detector (Shimadzu Corporation); injection volume: 100 μL. The sample was prepared at a final concentration of 1% (w w^{-1}) in distilled water. Standard P-82 (Showa Denko, Tokyo, Japan), maltose, and glucose (Kishida Chemical, Osaka, Japan) were used as standards to make a calibration curve.

The α- and β-anomers were distinguished, and the proportions of anomers were determined at 500 MHz ^{1}H NMR spectra and a probe temperature of 80°C, measured on the JEOL JNM-ECA500 spectrometer.[17] A total of 10 mg of dietary fiber was dissolved in 0.6 mL D_2O. The chemical shifts were expressed in ppm relative to sodium 3-(trimethylsilyl)-1-propanesulfonate sodium salt as an internal standard. From the spectra of 1H and 13C NMR, signals for the α-anomeric atoms were observed at δH 4.9–5.5 ppm and δC 95–100 ppm, while the β-anomeric atoms were detected at δH 4.4–4.7 ppm and δC

103–105 ppm. The ratio of the α to β anomers was determined using the integration ratio of the 1H NMR signals.

2.2 Collecting human fecal samples from volunteers

Fecal samples were obtained from eight healthy Japanese human volunteers who had not been treated with antibiotics for more than two months before participating in this study. All participants were recruited according to the following inclusion criteria: age ranging from the early twenties to the middle fifties, being Japanese, a non-smoking status, eating meals three times a day, and being in good health and good physical condition. The exclusion criteria included significant clinical deviation from normal as determined by the investigators: a history or suspicion of diabetes, liver disease, or kidney disease; having a food allergy; or currently taking prebiotics, probiotics, or lipid-lowering medications. Fecal samples were collected with an anaerobic culture swab (212550 BD BBL Culture Swab; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and used within 48 h.

2.3 Constructing the KUHIMM, with or without dietary fiber

The KUHIMM was employed with or without added prebiotics, using a multi-channel fermenter (Bio Jr.8; ABLE, Tokyo, Japan), as described in detail previously.[8,12] The 130 mL medium was based on the Gifu anaerobic medium (GAM [Code 05422]; Nissui Pharmaceutical Co, Tokyo, Japan) and was buffered to a pH of 6.5 by adding phosphate buffer (0.1 M NaH_2PO_4: 0.1 M Na_2HPO_4 = 2:1). Vessels containing the medium were constantly bubbled with an oxygen-free gas mixture (N_2:CO_2=80:20). The cultivations were initiated by inoculating a single fecal suspension (100 μL) into each fermenter vessel. During cultivation at 37 °C, the culture broth was stirred at 300 rpm with a magnetic stirrer and continuously purged with a filter-sterilized mixture of gas to maintain anaerobic conditions. To evaluate the degradation of dietary fiber, one type of dietary fiber

was added into one of the vessels at a final concentration of 10 g/L (1.0% per 100 mL medium) at 18 h after initiating cultivation. Aliquots of the culture broth were sampled from the vessels at 18, 21, 24, 27, 30, and 42 h after initiating the cultivation. Fecal and culture broth samples were stored at –20 °C until use.

2.4 Extraction of microbial genomic DNA

Microbial genomic DNA was extracted from suspended feces and culture broth from the KUHIMM at 42 h, as described previously.[18] Purified DNA was eluted into the TE buffer (10 mM Tris·HCl containing 1.0 mM ethylenediaminetetraacetic acid) and stored at –20 °C until use.

2.5 Illumina library generation

Bacterial 16S rRNA genes (V3–V4 region) were amplified using genomic DNA as the template and the primers S-D-Bact-0341-b-S-17 (5′-CCTACGGGNGGCWGCAG-3′) and S-D-Bact-0785-a-A-21 (5′-GACTACHVGGGTATCTAATCC-3′),[19] as described previously.[20] Index primers (Nextera XT Index Kit; Illumina Inc., San Diego, CA, USA) overhanging the amplified sequences were added to the gene-specific sequences. Each polymerase chain reaction (PCR) was performed according to the manufacturer's instructions. Amplicons were purified with AMPure XP DNA purification beads (Beckman Coulter, Brea, CA, USA) and eluted in 25 μL of 10 mM Tris (pH 8.5). Purified amplicons were quantified using an Agilent Bioanalyzer 2100 instrument with DNA 1000 chips (Agilent Technology) or a Qubit 2.0 fluorometer (Thermo Fisher Inc., Waltham, MA, USA) and then pooled at an equimolar concentration of 5 nM. The 16S rRNA genes and an internal control (PhiX control v3; Illumina) were subjected to paired-end sequencing using a MiSeq instrument (Illumina) and the MiSeq Reagent Kit, v3 (600 cycles; Illumina). The PhiX sequences were removed, and paired-end reads with Q scores ≥ 20 were joined using the MacQIIME

177 software package, version 1.9.1.[21] The UCLUST algorithm[22] was used to cluster the
178 filtered sequences into operational taxonomic units (OTUs) based on a ≥ 97% similarity
179 threshold. Chimeric sequences were checked and removed from the library using
180 ChimeraSlayer.[23] Representative sequences from each OTU were taxonomically classified
181 via the GreenGenes taxonomic database using the Ribosomal Database Project
182 Classifier.[24]
183
184 **2.6 Real-time PCR analysis**
185 Real-time PCR was performed to quantify total bacterial growth during cultivation, using a
186 LightCycler 96 system (Roche, Basel, Switzerland) with a primer set targeting all
187 eubacteria.[25,26] PCR amplification was performed as described previously.[18]
188
189 **2.7 Measurement of SCFA concentrations**
190 The concentrations of SCFAs such as acetate, propionate, butyrate, lactate, and succinate
191 were measured using a HPLC instrument (Shimadzu Corporation) equipped with an
192 Aminex HPX-87H column (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and a RID-10A
193 refractive-index detector (Shimadzu Corporation). The HPLC instrument was operated at
194 65 °C using 5 mM H_2SO_4 as the mobile phase, with a flow rate of 0.6 mL/min.
195
196 **2.8 Measurement of dietary fiber**
197 Aliquots of culture broth (5 mL) were mixed with phosphate buffer (15 mL, 0.08 M, final
198 pH 6.0). The indigestible contents were determined using enzymes in the Total Dietary
199 Fiber Assay Kit (Sigma, TDF-100A), following the procedure described in the AOAC Official
200 Method 2001.03.[27] The samples were gelatinized with heat-stable α-amylase and then
201 enzymatically digested with protease and amyloglucosidase to remove the proteins and
202 starch molecules present in the samples. Amberlites IRA67 and 200CT(H)HG were used to

203 remove various residual impurities. The indigestible content of dietary fiber was
204 quantified using HPLC with two TSKgel G2500PWxl columns (TOSOH, Japan).
205
206 **2.9 Bioinformatics and statistical analyses**
207 The α-diversity value (Shannon–Wiener index) was calculated from the OTU numbers
208 using the MacQIIME software package.[21] Principal coordinate analysis was conducted
209 using the OTU information for each sample and calculated based on unweighted UniFrac
210 distances,[28] using MacQIIME. Statistical analyses were performed using the Prism 8
211 software (GraphPad Software, Inc., San Diego, CA) and/or JMP 13 software (SAS Institute
212 Inc., Cary, NC, USA). $p < 0.05$ was considered to reflect a statistically significant difference.
213
214 **2.10 Data availability**
215 All raw sequence data generated in this study were deposited on the MG-RAST server[29]
216 (http://metagenomics.anl.gov) in a file named "Model Culture System of Human Colonic
217 Microbiota_Dietary fibers" under accession numbers mgm4884027.3–mgm4884090.3.
218
219 **3 Results**
220 **3.1 Establishment of a method for evaluating the fermentability of different dietary**
221 **fiber-containing glycosidic linkages**
222 Six types of dietary fibers were prepared, including PDX, RGN, DEX-1, DEX-2, DEX-3, and
223 IMD. Their glycosidic linkage contents were measured (Table 1). The contents of (1 → 2)
224 plus (1 → 3) linkages ranged from 1.4 to 20.7%, being lowest in IMD and highest in PDX.
225 To evaluate the fermentability of dietary fiber, we determined the appropriate
226 time to add the fiber to the *in vitro* KUHIMM. IMD was employed as its low (1 → 2) plus (1
227 → 3) linkages and β-linkage content exhibited high fermentation speed, as described
228 below. The microbial levels in the KUHIMM were measured by quantitative PCR

(Supporting Information, Fig. S1). The amount by which the microorganisms proliferated in the model culture system was near the maximum at 18 h after inoculating one fecal sample. The IMD-digestion rates were 0.48, 0.86, and 0.91 $g \cdot L^{-1} \cdot h^{-1}$ when IMD was added after 12, 18, and 24 h of fermentation, respectively. The IMD-digestion rate was underestimated if IMD was added before 18 h of fermentation. Thus, each dietary fiber was added after 18 h to evaluate fermentability.

3.2 Unaltered bacterial composition in the KUHIMM upon addition of dietary fibers

Each dietary fiber was administrated 18 h after constructing KUHIMMs from one of the eight different fecal inoculums. DNA was extracted from human feces and KUHIMM cultures at 24 h after adding 1.0% dietary fibers, i.e., after 42 h of fermentation. The eubacterial copy numbers (evaluated by quantitative PCR) reached up to $3.78 \pm 1.43 \times 10^{11}$ copies/mL in the KUHIMM cultures without dietary fiber addition (Supporting Information, Fig. S2) and were not significantly changed by adding any of the six different types of dietary fiber (paired t-test, $p > 0.05$).

Using the Illumina MiSeq platform, bacterial 16S rRNA gene sequencing was performed on eight fecal inocula and the corresponding KUHIMMs, with or without one of six different dietary fibers (PDX, RGN, DEX-1, DEX-2, DEX-3, and IMD) after 42 h of fermentation (Fig. 1). When comparing each fecal inoculum (FEC) and the KUHIMM without fiber addition (CUL), an average of 852 OTUs was found in the CUL, with an average of 1495 OTUs in the FECs. The biodiversity indices (such as the Shannon–Wiener and Simpson indexes) of the CUL were significantly decreased compared to those of the FECs (Wilcoxon's matched-pairs signed-rank test, $p < 0.05$). Therefore, it was considered that the number of bacterial species and the bacterial diversity in fecal inoculum were approximated in the KUHIMM. No significant differences were found between the

KUHIMMs without fiber addition and the KUHIMMs with fiber additions (Wilcoxon's matched-pairs signed-rank test, $p > 0.05$).

The KUHIMMs contained bacterial genera belonging to the Actinobacteria, Bacteroidetes, Firmicute, Fusobacteria, Proteobacteria, and Verrucomicrobia phyla, which were also observed in the FECs (Fig. 1E). The relative abundance of each bacterial genus in the CUL did not significantly differ ($p > 0.05$) from that in the KUHIMM with the addition of 1.0% dietary fiber (PDX, RGN, DEX-1, DEX-2, DEX-3, or IMD), as determined using Wilcoxon's matched-pairs signed-rank test (Fig. 1E and Supporting Information, Fig. S3).

3.3 Differences in the fermentability and SCFA production of dietary fibers in the KUHIMM

The fermentation speeds ($g \cdot L^{-1} \cdot h^{-1}$) of the indigestible part of each dietary fiber by the KUHIMM were determined during the period ranging from 18 h to 42 h of fermentation (Fig. 2A). In addition, the fermentation ratio (%) of the indigestible part of each dietary fiber at 42 h fermentation was determined (Fig. 2B). Both the fermentation speed and fermentation ratio were low for PDX and RGN, indicating that they were the most resistant to human colonic bacterial degradation among the six dietary fibers tested. In contrast, the fermentation speed and fermentation ratio were highest for IMD, indicating that it was most readily digested by colonic bacteria. Fermentability was ranked as follows: PDX = RGN < DEX-1 = DEX-2 < DEX-3 < IMD.

The SCFA concentrations in the KUHIMM culture were determined after 42 h of fermentation (Fig. 2C–E). As expected, adding PDX or RGN to the KUHIMM did not increase the production of acetate, propionate, or butyrate. However, adding DEX-1 and DEX-2 increased acetate production, and adding DEX-3 and IMD increased the production of acetate and propionate. Butyrate production was not affected by adding any of the dietary fibers to the KUHIMM.

3.4 Glycosidic linkage types correlate with dietary fiber fermentability

Here, we studied factors that can affect the digestibility of dietary fibers. The relationships between the abundances (%) of glycosidic linkages (1→2 plus 1→3) in dietary fiber and the average fermentation speeds ($g \cdot L^{-1} \cdot h^{-1}$), as well as the fermentation ratios (%) are shown in Fig. 3. Interestingly, these linkages were highly negatively correlated with the fermentability of the six different types of dietary fibers studied here (R^2 = 0.8126 or 0.8306, respectively). In addition, the abundance (%) of β-linkage was highly negatively correlated with the fermentability of the six different types of dietary fibers (R^2 = 0.9106 or 0.9673, respectively).

We then evaluated the relationship between SCFA production and the fermentation speed or fermentation ratio of dietary fibers (Fig. 4). When studying acetate, propionate, and butyrate production, we found that propionate production was most highly and positively correlated with the fermentation speed or ratio.

4 Discussion

Differences in the chemical structures of dietary fibers can impact microbial fermentation.[4] In our model culture system, the microbial abundances reached a steady state at 18 h after the initiation of fermentation, and the composition of a previously described microbial population was stable after 24 h of fermentation.[9] Thus, we selected 18 h as the optimal time for adding dietary fiber. As a result, we were able to compare the fermentation speeds and fermentation ratios at 24 h after adding different dietary fibers. We administrated dietary fiber to each *in vitro* model obtained from human volunteers, of whom ages ranged from the early twenties to the mid-fifties, since the human gut microbiota composition changes with age.[30] Furthermore, the species richness observed in the KUHIMM *in vitro* model decreased compared to the original fecal inoculum;

306 however, our previous model maintained species richness.[8,9] This discrepancy was due to
307 the addition of a buffer in the current study to maintain the pH and measure the
308 fermentation rate under conditions that most closely mimic the pH (~6.5) of the colon.[31]
309 Our *in vitro* model for investigating the human colonic microbiota, i.e., the
310 KUHIMM, showed that the content of (1 → 2) plus (1 → 3) linkages and β-linkage
311 negatively correlated with the fermentability of dietary fiber. This result indicates that (1
312 → 2) plus (1 → 3) linkages and β-linkage, which are newly formed during the
313 modification of starch or glucose, would determine recalcitrance against bacterial
314 degradation.[13] The dietary fibers tested here contained different types of linkages, and
315 various enzymes such as polysaccharidases and glycosidases would be necessary to
316 metabolize them. The Bacteroidetes phylum, composed largely of members of the
317 *Bacteroides* genus, exhibited broad capacities in metabolizing a variety of plant-derived
318 glycans, whereas other microbes could only utilize one or a few different
319 polysaccharides.[32] *Bacteroides thetaiotaomicron* and *Bacteroides ovatus* are human gut
320 microbiota known to utilize more than a dozen different types of glycans.[33] Our results
321 correspond with those of a previous report showing that α-(1 → 2) glycosidic linkages in
322 α-gluco-oligosaccharides were more resistant than α-(1 → 6) linkages used by *B.*
323 *thetaiotaomicron.*[34]
324 In humans, SCFAs exert multiple beneficial effects, such as normalizing plasma
325 glucose levels and lowering plasma cholesterol.[35] Here, the high fermentability of dietary
326 fiber correlated with high SCFAs production, such as acetate and propionate. This result
327 was reasonable, considering that these SCFAs are generated by colonic microbiota, as the
328 result of dietary fiber fermentation. Acetate comprised a major fraction of the total SCFAs,
329 which was expected, as many of the bacterial groups that inhabit the colon can generate
330 acetate.[36] More importantly, propionate production was highly affected by the
331 fermentability of dietary fiber. For propionate formation, the first pathway suggests that a

route related to succinate was generally employed by *Bacteroides* species, whereas another pathway (the acrylate pathway route starting with lactate) was adopted by bacteria belonging to the clostridial cluster IX group.[37] As supported by Figs. S2 and S4 in the Supporting Information section, *Bacteroides* species could be responsible for both dietary fiber fermentation and propionate production.

In conclusion, we developed an *in vitro* human colonic microbiota model (the KUHIMM) to measure the fermentability of dietary fiber. A limitation of this study may be the difficulty of distinguishing sugar in the medium compared to that produced from dietary fiber. We demonstrated that the contents of (1 → 2) plus (1 → 3) linkages and β-linkage in dietary fiber affected the fermentability. Based on information related to glycosidic linkage structures of dietary fibers, we could expect SCFA productivity. In the future, KUHIMM can be applied to evaluate gut microbe-drug interactions,[38] as well as the effect of engineered commensal microbes.[39]

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

Authors' contributions

DS and KS conceived the study and designed the experiments. DS performed the NGS analyses. KS and DS wrote the manuscript. AK designed and supervised the study. All authors read and approved the final manuscript.

Declarations

Ethics approval and consent to participate

All subjects provided written informed consent before specimen collection. The study was performed in accordance with the guidelines of Kobe University Hospital and was approved by the institutional ethics review board of Kobe University (research code, 1902; approval date, May 10, 2016). All methods used in this study were performed in accordance with the Declaration of Helsinki. The authors have no financial or personal relationships that could inappropriately influence this research.

Consent for publication

Not applicable

Availability of data and material

All raw sequence data generated in this study were deposited on the MG-RAST server[29] (http://metagenomics.anl.gov) in a file named “Model Culture System of Human Colonic Microbiota_Dietary fibers” under accession numbers mgm4884027.3–mgm4884090.3.

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482 **Table 1. Dietary fibers used in this study.**

Dietary fiber	Glycosidic linkages (%)					Molecular weight	Indigestible part (%)[a]
	1→4	1→6	1→2	1→3	β / α		
Polydextrose (PDX)	25.2	54.0	12.8	7.9	0.44	728	82.2
Resistant glucan (RGN)	35.2	48.0	10.6	6.2	0.46	792	77.3
Indigestible dextrin (DEX-1)	53.4	34.3	5.7	6.7	0.39	2190	88.3
Indigestible dextrin (DEX-2)	38.3	47.5	8.2	6.0	0.42	1198	90.8
Indigestible dextrin (DEX-3)	51.5	36.8	5.1	6.6	0.31	1371	96.7
Isomaltodextrin (IMD)	26.8	71.8	0.0	1.4	0.06	2176	87.0

483

484 [a] Dry-weight basis

485

486

Figure legends

Graphical abstract

An *in vitro* model of human colonic microbiota can be used to efficiently evaluate the fermentability of dietary fibers. The *in vitro* model clarified the relationship between the glycosidic linkage content of dietary fiber and their fermentability. In addition, this model showed the relationship between propionate production and fermentability.

Figure 1. Bacterial 16S rRNA gene sequencing. 16S rRNA genes amplified using DNA extracted from FECs, CULs, or KUHIMMs, with the addition of 1.0% dietary fiber (RGN, PDX, DEX-1, DEX-2, DEX-3, or IMD), after 42 h of fermentation were evaluated. A) Numbers of quality-controlled sequences obtained by NGS. B) Observed numbers of OTUs. C) Shannon–Wiener index. D) Simpson index. * Significant differences (*$p < 0.05$ and **$p < 0.01$) from the FEC values (n = 8, designated as HS1–HS8), as determined using Wilcoxon's matched-pairs signed-rank test. The data shown are presented as medians and interquartile ranges (25th–75th percentiles). E) Genus-level compositional view. Genera with a low abundance (<1.0%) and a low similarity (<97%) were included in the "others" and "unclassified bacteria", respectively.

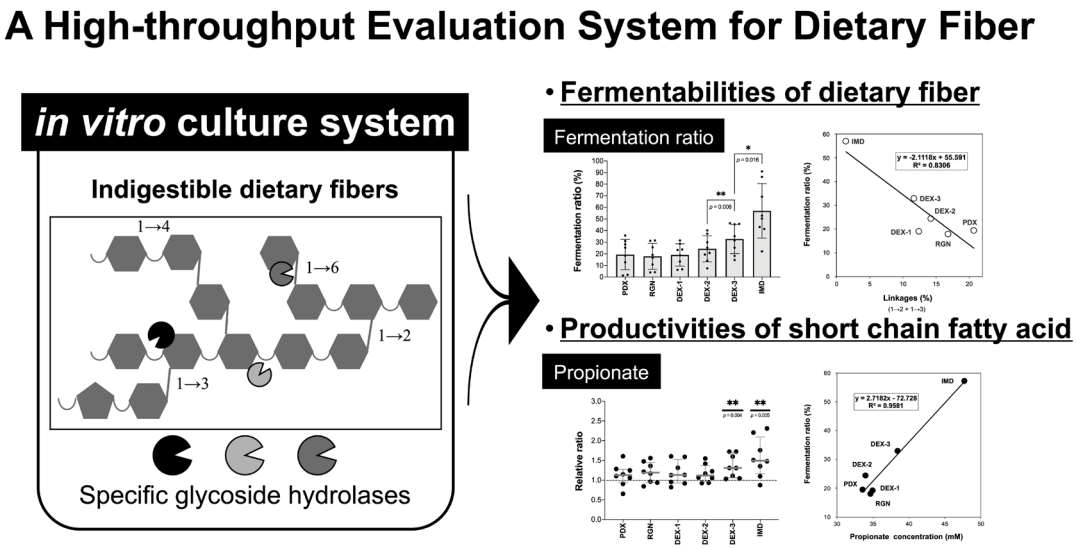
Figure 2. Fermentability of the indigestible part of 1.0% dietary fiber (RGN, PDX, DEX-1, DEX-2, DEX-3, or IMD). A) The fermentation speed ($g \cdot L^{-1} \cdot h^{-1}$) was calculated using linear fermentation values. The data are presented as medians and interquartile ranges (25th–75th percentiles). B) The fermentation ratio (%) was calculated as follows: (indigestible amount of dietary fiber after 42 h of fermentation)/(initial indigestible amount of dietary fiber after 18 h of fermentation). Data are presented as means and standard deviations. *Significant differences (*$p < 0.05$ and **$p < 0.01$) between dietary fibers using Wilcoxon's matched-pairs signed-rank test. Changes in the production of C) acetate, D) propionate,

and E) butyrate after 42 h of fermentation are shown. The changes are presented as the ratio of the concentration in the KUHIMM culture supplemented with dietary fiber normalized to that in the control (without dietary fiber). The control system generated acetate, propionate, and butyrate at mean concentrations (± SD, n = 8) of 90.3 ± 15.0, 28.9 ± 8.95, and 13.5 ± 12.0 mM, respectively. *Significant differences (*$p < 0.05$ and **$p < 0.01$) from the control values, as determined using Dunnett's test. Data are presented as medians and interquartile ranges (25th–75th percentiles).

Figure 3. Relationships between the percentages (%) of (1→2) plus (1→3) linkages or β-linkage and the average A or C) fermentation speed ($g \cdot L^{-1} \cdot h^{-1}$) or B or D) fermentation ratio (%) of the indigestible parts of 1.0% dietary fibers (RGN, PDX, DEX-1, DEX-2, DEX-3, or IMD). The solid line and the corresponding line equation indicate the best-fit linear relationship.

Figure 4. Relationships between the average SCFA concentrations and average fermentation speed or fermentation ratio of the indigestible part of 1.0% dietary fibers (RGN, PDX, DEX-1, DEX-2, DEX-3, or IMD). A–C) Relationships between the fermentation speed ($g \cdot L^{-1} \cdot h^{-1}$) and average acetate, propionate, and butyrate concentrations (mM), respectively. D–F) Relationships between the fermentation ratio (%) and the average acetate, propionate, and butyrate concentrations (mM), respectively. The solid line and the corresponding line equation indicate the best-fit linear relationship.

535 **Graphical abstract**



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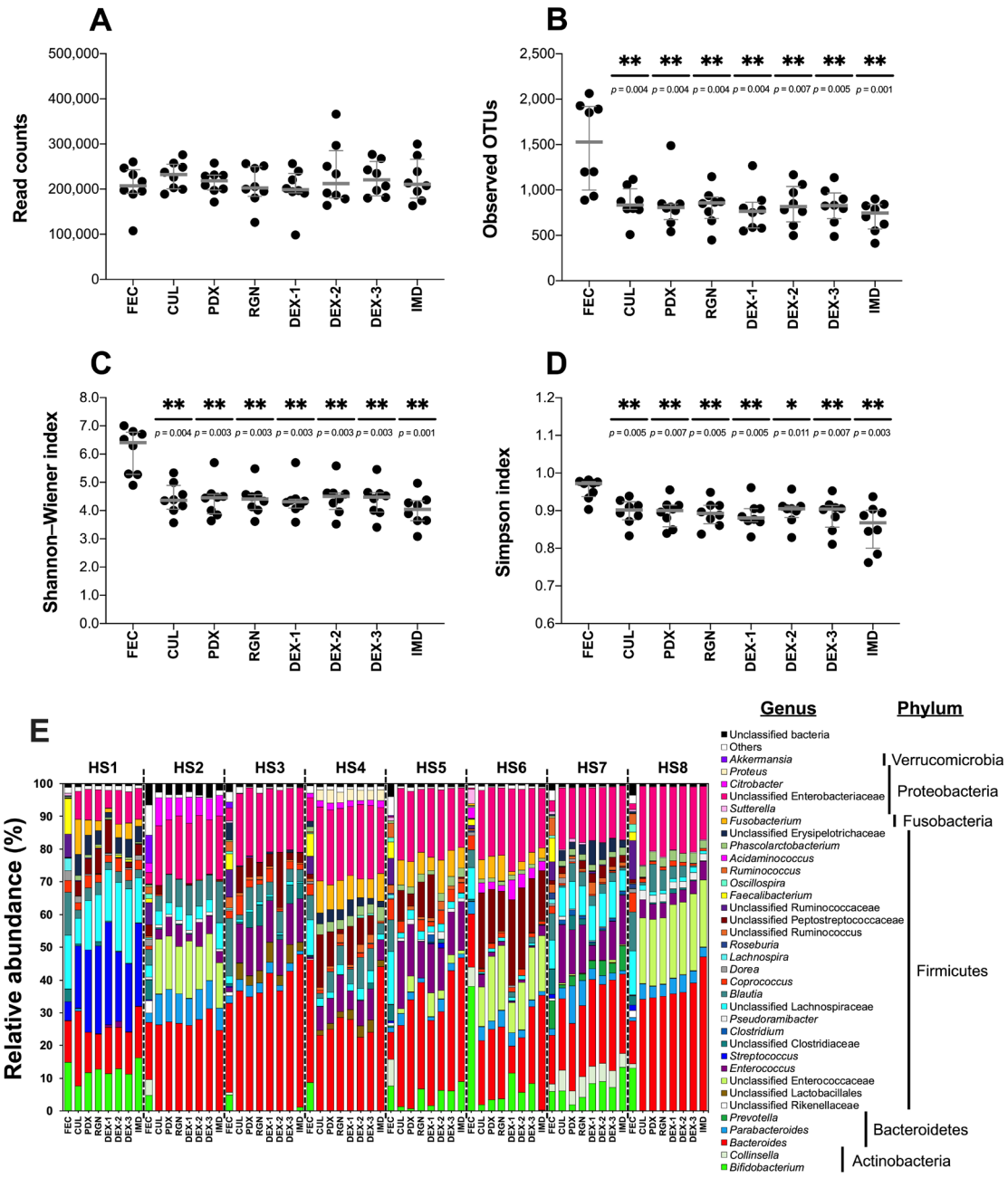
539 **Figure 1.**



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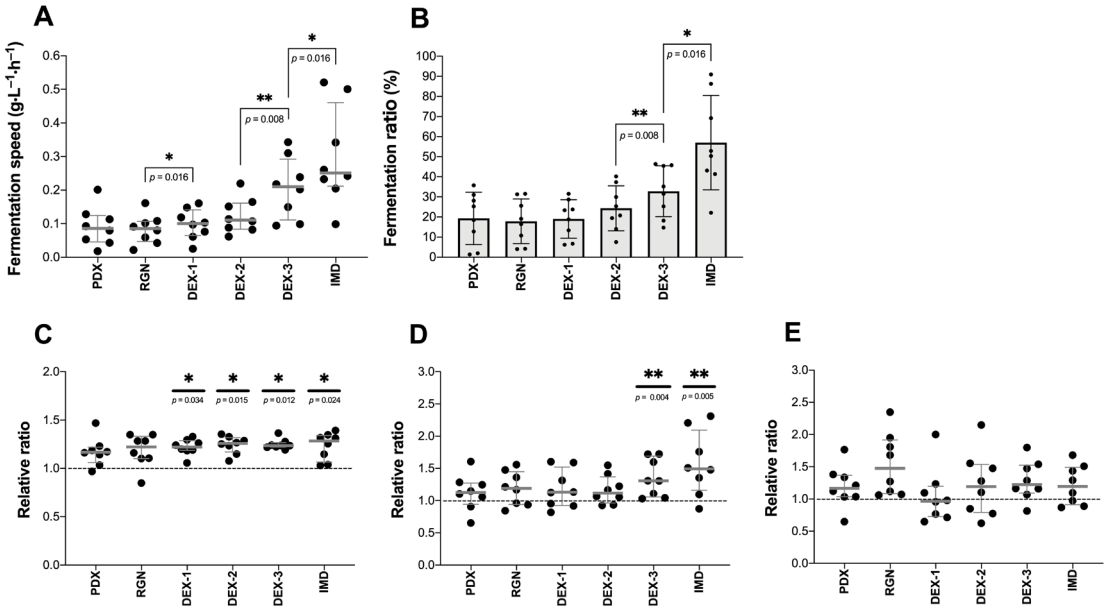
542 **Figure 2.**



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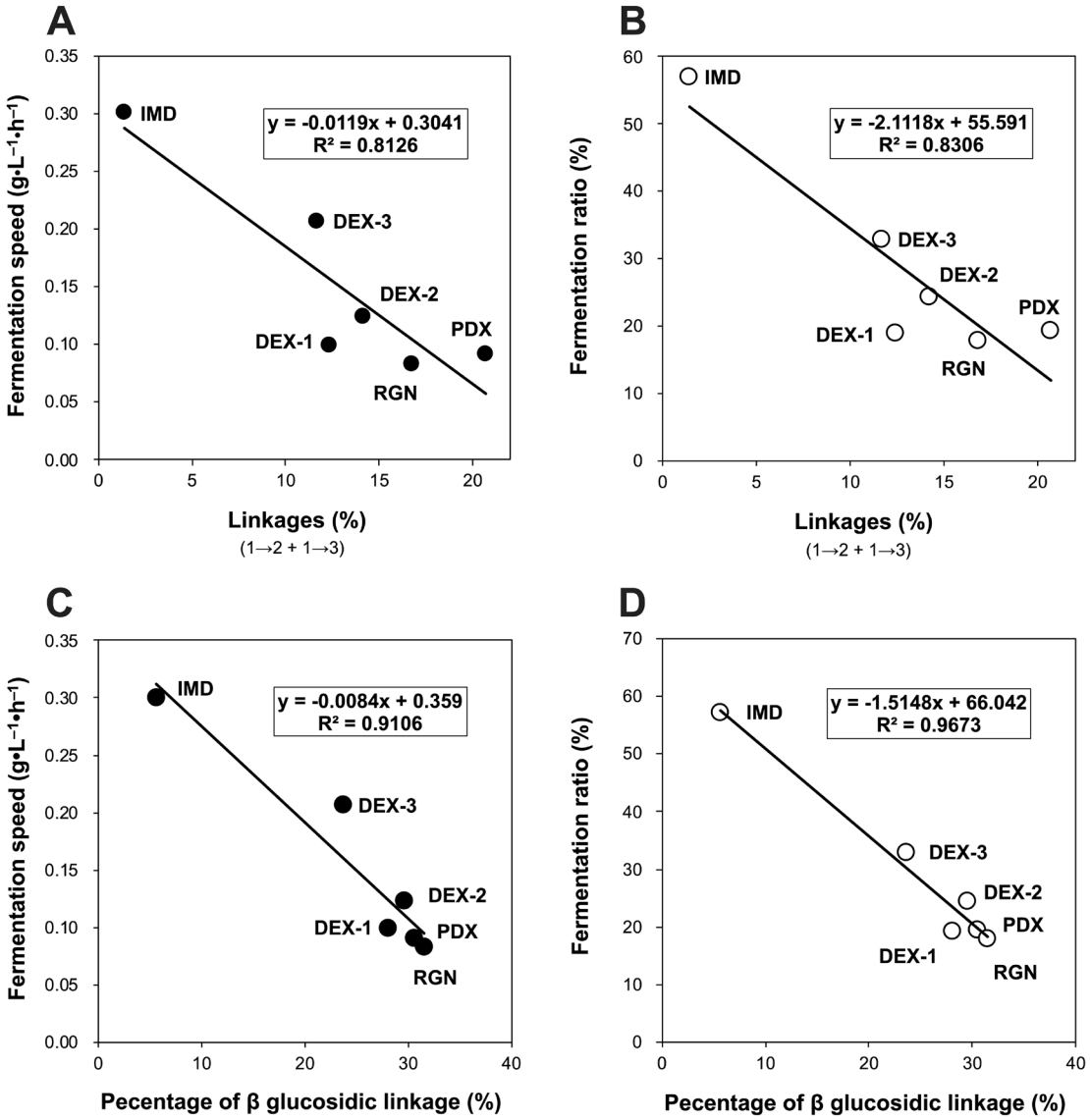
545 **Figure 3.**



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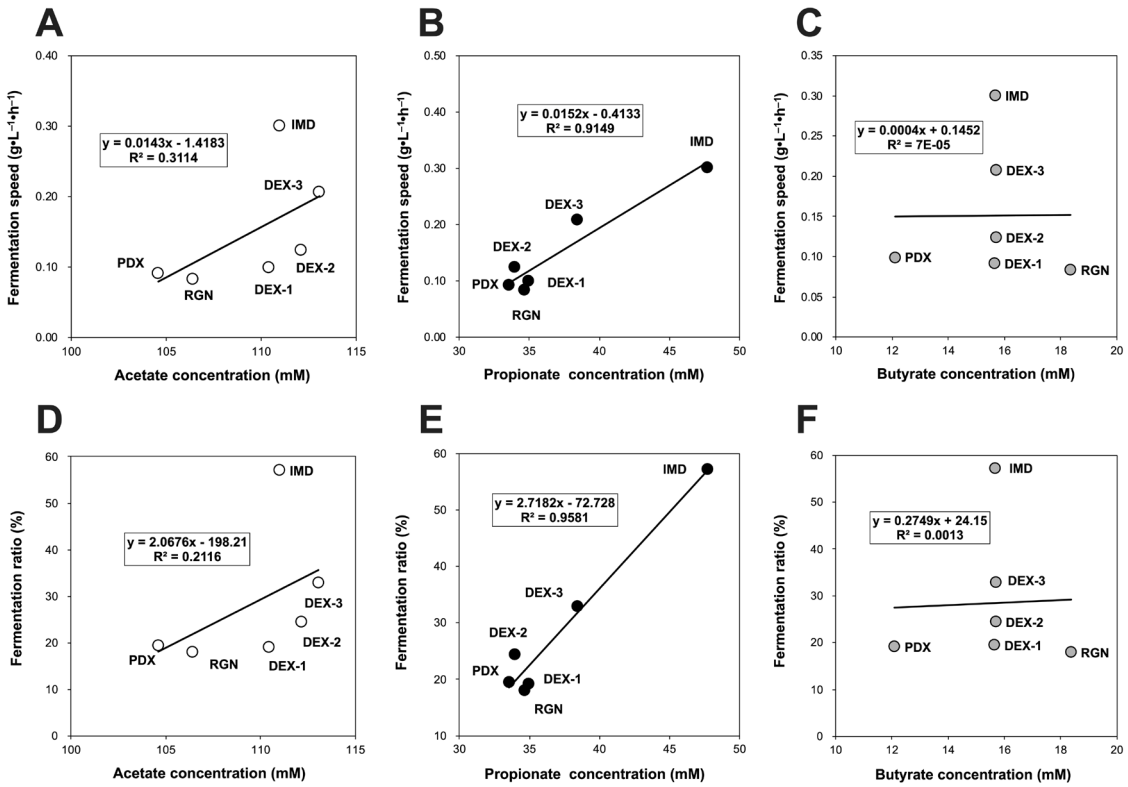
548 **Figure 4.**



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Supporting Information

Research Article

Glycosidic linkage structures influence dietary fiber fermentability and propionate production by human colonic microbiota *in vitro*

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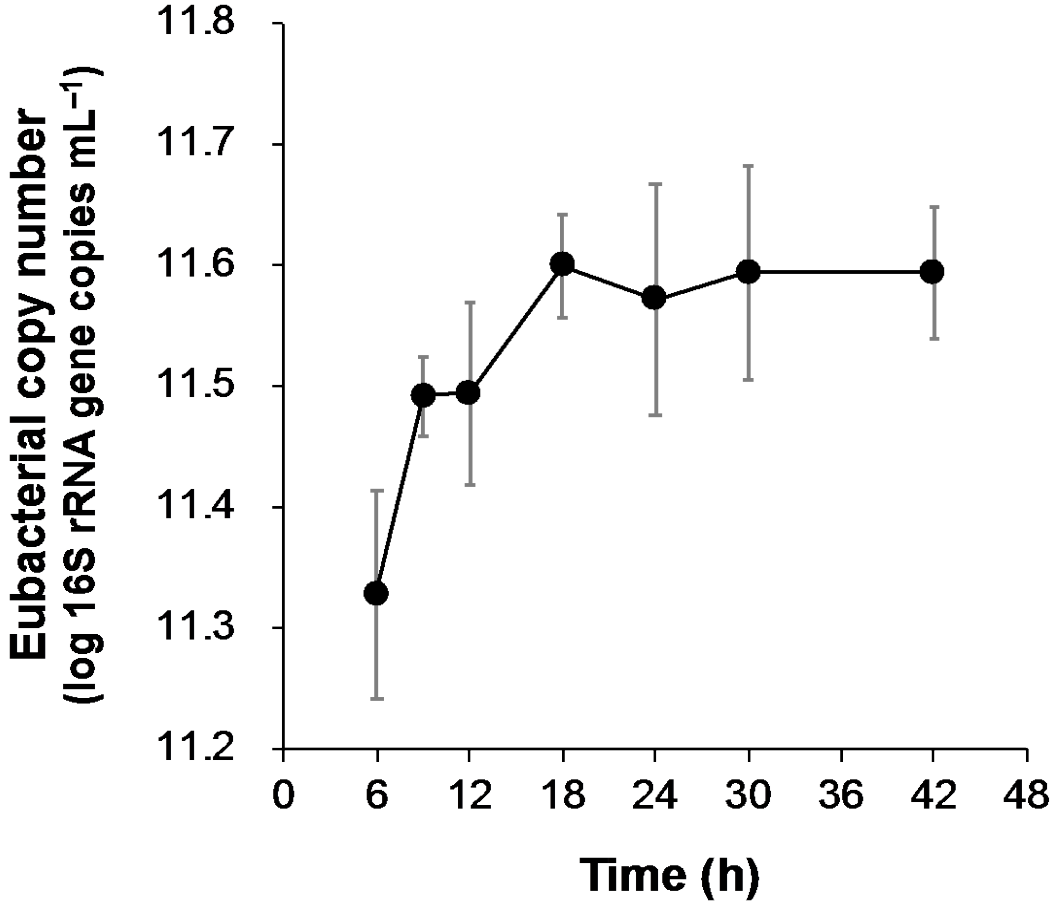
Eubacterial copy number
(log 16S rRNA gene copies mL^{-1})
11.8
11.7
11.6
11.5
11.4
11.3
11.2
0
6
12
18
24
30
36
42
48
Time (h)

570

571

572 **Supporting Information Figure S1.**

573 Copies of eubacteria (all types of bacteria except for archaeabacteria) in our *in vitro* human colonic
574 microbiota model (KUHIMM) were calculated in triplicates using quantitative PCR after 6, 9, 12, 18, 24,
575 30, and 42 h of fermentation.

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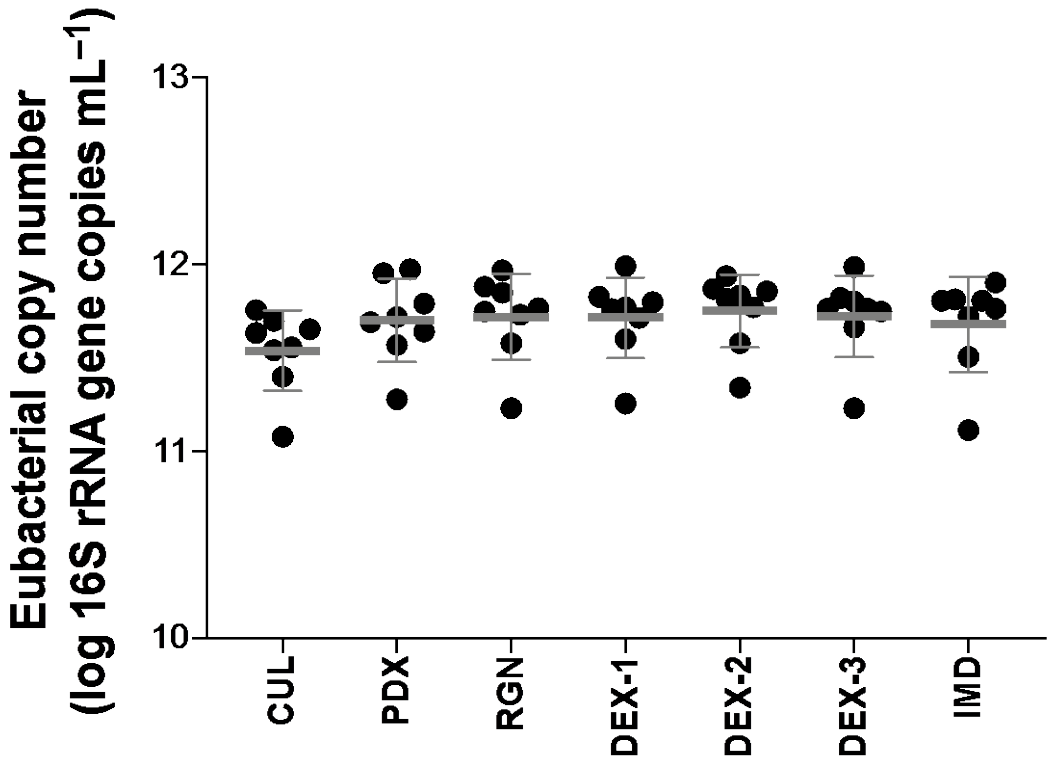

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579 **Supporting Information Figure S2.**

580 Eubacterial copy numbers in our *in vitro* human colonic microbiota model (KUHIMM) cultures
581 without (CUL) or with one of 6 different types of 1.0% dietary fiber (RGN, PDX, DEX-1, DEX-2, DEX-
582 3, or IMD) were quantified after 42 h of fermentation (i.e., at 24 h after dietary fiber addition). The
583 copy numbers (n = 8) in CUL samples were not significantly different ($p > 0.05$) from those
584 observed after adding any dietary fiber, as determined using a paired t-test.

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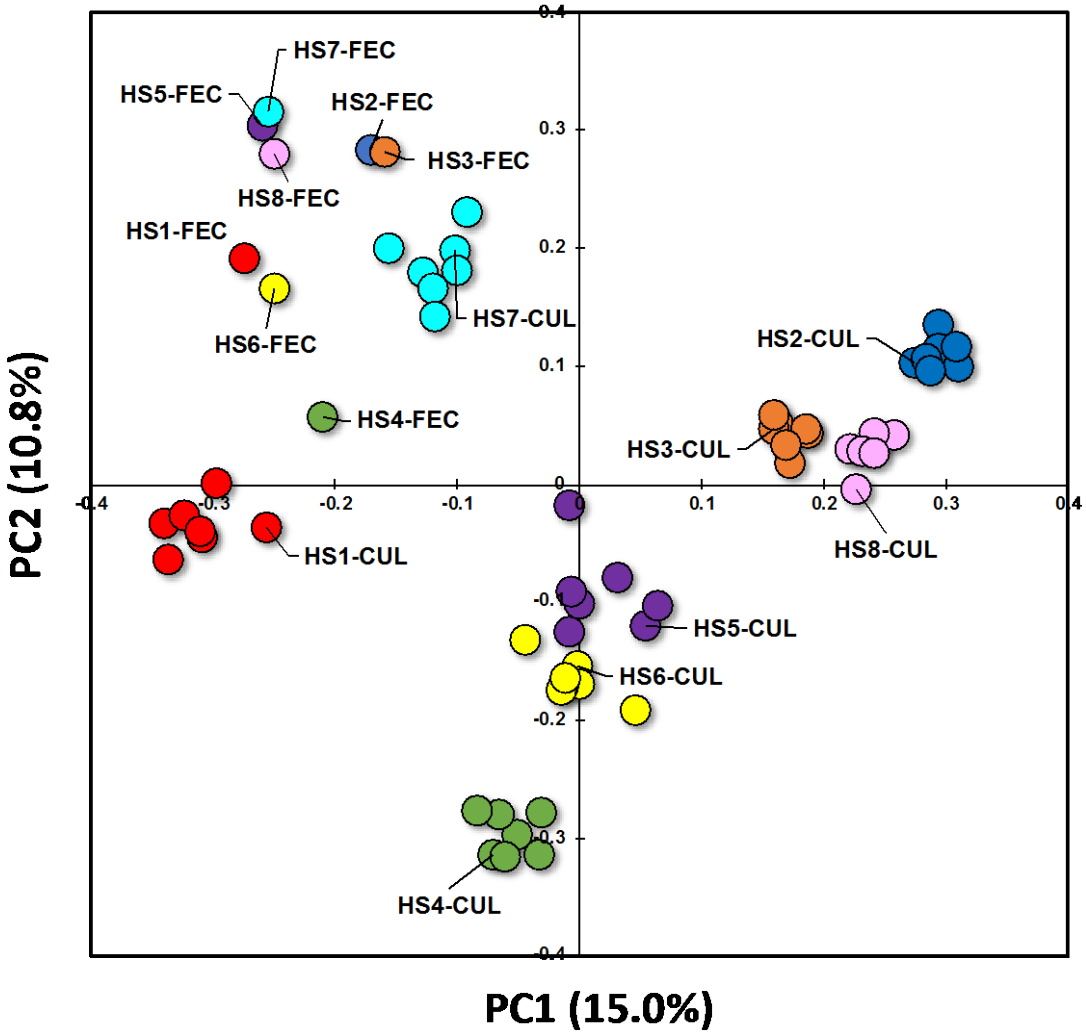

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588 **Supporting Information Figure S3.**

589 PCoA of 16S rRNA gene sequence data for the bacterial species present in fecal samples from eight
590 human volunteers (designated as HS1-FEC to HS8-FEC) and in the corresponding cultures with and
591 without 1.0% dietary fiber. KUHIMM cultures were sampled after 42 h of fermentation, i.e., 24 h
592 after the addition of 1.0% dietary fiber.

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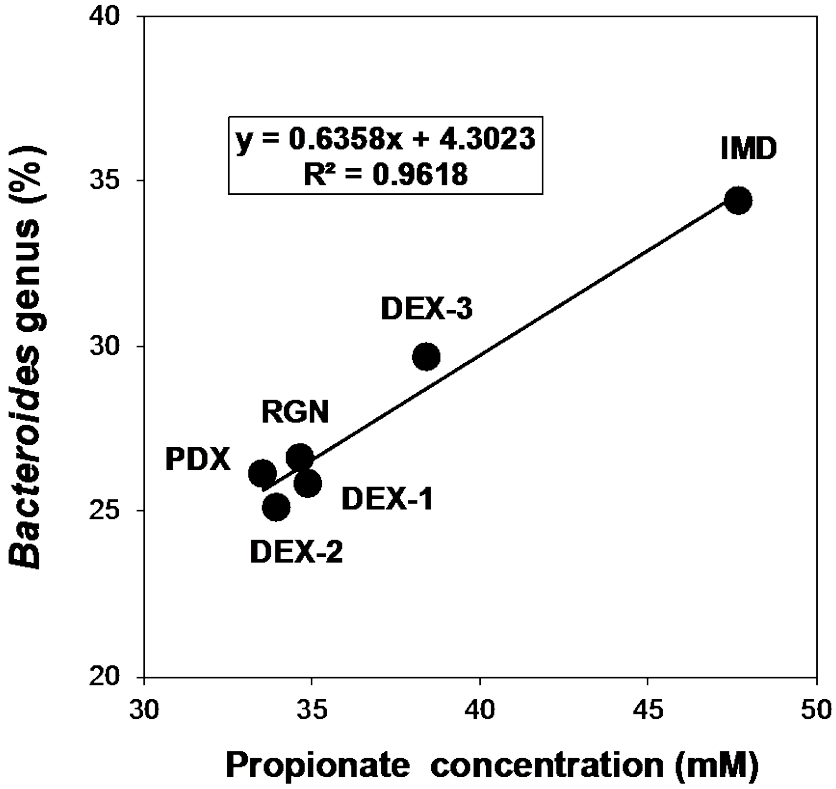

Supporting Information Figure S4.

Relationships between the average propionate concentrations (mM) and average percentages (%) of *Bacteroides* genus in KUHIMMs with the addition of 1.0% dietary fibers (RGN, PDX, DEX-1, DEX-2, DEX-3, or IMD). The solid line and the corresponding line equation indicate the best-fit linear relationship.