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Effect of Resistant Starch on the Gut Microbiota and Its Metabolites in Patients with Coronary Artery Disease

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Aim: *Bacteroides vulgatus* and *B. dorei* have a protective effect against atherosclerosis, suggesting that expansion of these species in the gut microbiota could help patients with coronary artery disease (CAD). This study aimed to investigate the effect of resistant starch (RS) on the gut microbiota and its metabolites in fecal sample cultures from patients with CAD and individuals without CAD, using a single-batch fermentation system.

Methods: Fecal samples from 11 patients with CAD and 10 individuals without CAD were fermented for 30 h with or without RS in the Kobe University Human Intestinal Microbiota Model (KUHIMM). Gut microbiota and the abundance of *B. vulgatus* and *B. dorei* were analyzed using 16S ribosomal ribonucleic acid (rRNA) gene sequencing and the quantitative polymerase chain reaction. Short-chain fatty acids were analyzed using high-performance liquid chromatography.

Results: Gut microbial analysis showed significantly lower levels of *B. vulgatus* and *B. dorei* in the original fecal samples from patients with CAD, which was simulated after 30 h of fermentation in the KUHIMM. Although RS significantly increased the absolute numbers of *B. vulgatus* and *B. dorei*, and butyrate levels in CAD fecal sample cultures, the numbers varied among each patient.

Conclusions: The effect of RS on gut microbiota and its metabolites in the KUHIMM varied between CAD and non-CAD fecal sample cultures. The KUHIMM may be useful for preclinical evaluations of the effects of RS on the gut microbiota and its metabolites.

Key words: Coronary artery disease, Gut microbiota, Resistant starch, *Bacteroides*

Introduction

Coronary artery disease (CAD) is the leading cause of morbidity and mortality worldwide. Despite appropriate therapeutic interventions to mitigate CAD risk factors, a residual risk of >50% still remains^{1, 2)}. Increasing evidence supporting a strong association between the gut microbiota and the pro-

gression of atherosclerotic cardiovascular diseases is accumulating³⁻⁷⁾. We published the first description of the relationship between the gut microbiota and CAD, and the findings of our study reported lower *Bacteroidetes*-to-*Firmicutes* ratios, lower levels of bacteria of the phylum *Bacteroidetes* and genus *Bacteroides*, and higher levels of bacteria of the order *Lactobacillales* in patients with CAD^{8, 9)}. In accordance with our

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results, individuals with low levels of bacteria of the genus *Bacteroides* have a higher incidence of atherosclerosis^{10, 11}). In addition, we have recently reported that *B. vulgatus* and *B. dorei* protect against atherosclerosis by ameliorating endotoxemia¹²). Because these findings may lead to the development of novel approaches for the prevention and treatment of CAD by modulating the components of the gut microbiota, recent studies have focused on non-invasive interventions that could modulate gut microbiota^{5, 13, 14}).

Each gut microbial profile depends on age, sex, local diet or lifestyle, drugs, and other many factors. These factors may relate to the progression of CAD via modulation of gut microbiota. The findings from many clinical trials and animal studies have shown that diet plays a central role in shaping the gut microbiota^{15, 16}). Undigested dietary fiber, in particular, alters the microbial ecosystem of the gut and its composition, because the gut microbiota uses dietary fiber as a primary energy source^{17, 18}). The findings from several human studies have shown higher *Bacteroidetes-to-Firmicutes* ratios and higher *Bacteroides* levels in people who consume high-fiber diets^{19, 20}). Although the findings from large epidemiological studies have consistently shown that intake of dietary fiber can prevent CAD in healthy subjects²¹⁻²³), the beneficial effects of altering the gut microbiota in patients with CAD remain unclear²⁴).

SCFAs, primarily acetate, propionate, and butyrate, are the end products of dietary fiber fermentation generated by gut microbiota, and these end products act as signaling molecules to regulate the metabolism and inflammatory responses of the host²⁵⁻²⁷).

Aim

This study aimed to determine whether undigested dietary fiber could alter the gut microbiota from patients with CAD to achieve a symbiotic state. We used a single-batch anaerobic fermentation system, namely, the Kobe University Human Intestinal Microbiota Model (KUHIMM), to explore our hypothesis²⁸⁻³⁰), because ethical considerations constrain human interventional clinical trials. The KUHIMM can simulate the human gut microbiota metagenomically and metabolically, and it facilitates evaluations of the effect of dietary fiber on the gut microbiota before clinical trials. Because undigested dietary fiber in the form of resistant starch (RS) increases the abundance of *Bacteroides* spp. in mice³¹), we fermented feces obtained from CAD patients with RS in the KUHIMM. We also fermented feces obtained from non-CAD control individuals with coronary risk factors, including hypertension, diabetes,

and dyslipidemia to compare the effects of RS on the gut microbiota between CAD patients and non-CAD control individuals.

Methods

Study Participants

Eleven patients with CAD comprised the study group and 10 individuals without CAD who had coronary risk factors comprised the non-CAD control group. Study participants were recruited at Kobe University Hospital between February 2017 and December 2017. Patients with heart failure; renal disease, which was defined as a serum creatinine concentration >2.0 mg/dL; hepatic disease; malignancies; or concomitant inflammatory conditions were excluded from the study. Patients who were treated with antibiotics during the 4 weeks before the study commenced were also excluded from our study. Written informed consent was obtained from all participants, and the study was conducted according to the principles of the Declaration of Helsinki. This study was approved by Kobe University's Ethics Committee (Approval no. 160191), and it was registered with the UMIN Clinical Trials Registry (Trial registration no. UMIN000024555).

Blood Sampling

Blood samples collected after an overnight fast were tested to determine the levels of aspartate aminotransferase, alanine aminotransferase, γ -glutamyl transpeptidase, C-reactive protein, blood urea nitrogen, creatinine, glycated hemoglobin, high-density lipoprotein, low-density lipoprotein (LDL), total cholesterol, triglyceride, and brain natriuretic peptide.

Fecal Samples and Culture With Resistant Starch

We used the KUHIMM, which is a small-scale multichannel fermenter (Bio Jr.8; ABLE, Tokyo, Japan) that comprises eight parallel and independent anaerobic culture vessels (**Supplementary Fig. 1**)²⁸⁻³⁰). A maximum of eight independent experiments can be performed, which uses all of the vessels. Each vessel contains 100 mL of Gifu anaerobic medium (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) at pH 6.5. The medium was maintained at 37°C and was stirred regularly at 300 rpm. Anaerobiosis was maintained by the continuous in-flow (15 mL/min) of a filtered gas mixture comprising 80% N₂ and 20% CO₂.

Fecal samples were collected during the time that the participants were hospitalized and thus consuming a hospital diet for more than 1 day. Hospital diet contains approximately 12 g/day of dietary fiber. Each fecal sample stored in an anaerobic culture swab trans-

port device (BBL™ CultureSwab™; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) was used within 24 h. The fecal samples were suspended in 2 mL of 0.1 M phosphate buffer at pH 6.5, which comprised a 2:1 mixture of NaH₂PO₄ and 0.1 M Na₂HPO₄ supplemented with 1% L-ascorbic acid (Wako Pure Chemical Industries, Osaka, Japan). One vessel was inoculated with 100 µL of a fecal suspension only (culture group). Immediately after the fecal inoculation, 0.5% RS (FIBOSE®; Nippon Starch Chemical Co., Ltd., Osaka, Japan) was added to some of the vessels.

Microbial Deoxyribonucleic Acid Extraction

Microbial genomic deoxyribonucleic acid (DNA) was extracted from fecal sample suspensions and the culture broths from the KUHIMM at 30 h, as described previously³⁰. The purified DNA was eluted into Tris-EDTA buffer, comprising 10 mM Tris-HCL and 1.0 mM EDTA, and was stored at -80°C until required.

Library Generation

Bacterial 16S ribosomal ribonucleic acid (RNA) genes from the 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'-GACTACH-VGGGTATCTAATCC-3')³², as described previously³⁰. Index primers (Nextera XT Index Kit; Illumina, Inc., San Diego, CA, USA) overhanging the amplified sequences were added to the gene-specific sequences. The polymerase chain reaction (PCR) preparation and assays were performed according to the manufacturer's instructions. The amplicons were purified using AMPure XP DNA purification beads (Beckman Coulter, Brea, CA, USA), and they were eluted in 25 µL of 10 mM Tris buffer (pH 8.5). The purified amplicons were quantified using an Agilent Bioanalyzer 2100 with DNA 1000 chips (Agilent Technology, Santa Clara, CA, USA) and Qubit 2.0 (Thermo Fisher Inc., Waltham, MA, USA), and they were pooled at equimolar concentrations (5 nM). The 16S ribosomal RNA genes and an internal control (PhiX control v3; Illumina) were subjected to 600 cycles of paired-end sequencing using a MiSeq® sequencer (Illumina, Inc.) and a MiSeq® reagent kit v3 (Illumina, Inc.). The PhiX sequences were removed, and paired-end reads with Q scores ≥ 20 were joined using MacQIIME software, version 1.9.1 (Werner Lab, Cortland, NY, USA)³³. The UCLUST algorithm³⁴ was used to cluster the filtered sequences into operational taxonomic units (OTUs) based on a ≥ 97% similarity threshold. The chimeric sequences

were checked and removed from the library using ChimeraSlayer³⁵. Representative sequences from each OTU were taxonomically classified using the Green-Genes taxonomic database and the Ribosomal Database Project Classifier³⁶.

Quantitative Real-Time Polymerase Chain Reaction

Quantitative real-time PCR analyses were performed using SYBER Premix Ex Taq (#RR820; Takara Bio, Inc., Shiga, Japan) and a LightCycler® 96 System (#05815916001; Roche Applied Science, Mannheim, Germany) according to the manufacturers' instructions. We used PCR primers to detect *B. vulgatus* and *B. dorei*, as described previously³⁷.

Short-Chain Fatty Acid Analysis

The levels of acetate, propionate, and butyrate were measured using a high-performance liquid chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with an Aminex HPX-87H column (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and an RID-10A refractive index detector (Shimadzu Corporation) at 65°C. The mobile phase comprised 5 mM H₂SO₄ at a flow rate of 0.6 mL/min.

Statistical Analyses

The statistical analyses were performed using JMP software, version 14 (SAS Institute, Cary, NC, USA), R version 3.1.0 (The R Foundation for Statistical Computing, Vienna, Austria; <http://cran.r-project.org>), MacQIIME (Werner Lab), and GraphPad Prism software, version 7.0 (GraphPad Software; La Jolla, CA, USA). The Shapiro–Wilk test was used to determine the normality of the data. The results are expressed as the means and the standard deviations for the normally distributed data, and as the medians and the interquartile ranges (25th–75th percentiles) for the non-normally distributed data. The differences in the continuous parameters between groups of unpaired data were analyzed using the two-tailed Student's *t*-test for the normally distributed data or the Mann–Whitney U-test for the non-normally distributed data. Comparisons of samples from the same patients were performed using the paired *t*-test or the Wilcoxon signed-rank test for matched pairs. The categorical variables were compared using Fisher exact test or the chi-squared test. The clustering of the data from the patients with CAD (*n*=11) and from the individuals who did not have CAD (*n*=10) was performed at the genus level, as previously described¹². The Shannon–Wiener index and the Simpson index were calculated using MacQIIME (Werner Lab). The principal component analysis was conducted using JMP software (SAS Institute). All statistical analyses

Table 1. Baseline characteristics of the study population

Variables	Non-CAD controls (<i>n</i> = 10)	CAD (<i>n</i> = 11)
Age (years)	63.4 ± 6.3	73.1 ± 10.4*
Sex, male (%)	7 (70)	10 (91)
BMI (kg/m ²)	24.3 ± 4.2	24.0 ± 3.1
Laboratory data		
AST (U/L)	27.1 ± 11.7	24.0 ± 4.3
ALT (U/L)	23.6 ± 12.5	22.5 ± 8.7
γ-GTP (U/L)	49.3 ± 38.2	30.7 ± 18.5
BUN (mg/dL)	15.3 ± 4.2	18.8 ± 3.9
Creatinine (mg/dL)	0.9 ± 0.2	0.9 ± 0.14
T-Cho (mg/dL)	199.7 ± 21.7	157.9 ± 39.8*
HDL-C (mg/dL)	55.7 ± 15.1	48.8 ± 15.1
LDL-C (mg/dL)	117.6 ± 17.5	82.9 ± 23.0**
TG (mg/dL)	181.8 ± 108.1	171.4 ± 201.7
HbA1c (NGSP%)	6.0 ± 0.7	6.6 ± 1.1
CRP (mg/dL)	0.05 ± 0.06	0.15 ± 0.16
BNP (pg/mL)	40.1 ± 38.2	45.9 ± 25.4
History of smoking, <i>n</i> (%)	6 (60)	4 (36)
Current smoker, <i>n</i> (%)	3 (30)	2 (18)
Past history, <i>n</i> (%)		
Diabetes Mellitus	2 (20)	5 (45)
Dyslipidemia	5 (50)	11 (100)*
Hypertension	5 (50)	9 (82)
Medications, <i>n</i> (%)		
ACE-I/ARB	4 (40)	6 (55)
Anticoagulant or Antiplatelet	6 (60)	10 (91)
β-blocker	2 (20)	2 (18)
Calcium channel blocker	6 (60)	4 (36)
Metformin	0 (0)	2 (18)
α-glucosidase inhibitor	0 (0)	1 (9)
PPI/H2 blocker	6 (60)	10 (91)
Statin	4 (40)	11 (100)**

Data are shown as mean ± standard deviation, or counts (%). The averages of continuous variables were compared using either the two-tailed student's *t* test. Fisher's exact test were used to compare the proportions of categorical variables between groups. A two-sided value of *p* < 0.05 was considered statistically significant. ACEI, angiotensin-converting enzyme inhibitor; ALT, alanine aminotransferase; ARB, angiotensin receptor blocker; AST, aspartate aminotransferase; BMI, body mass index; BNP, brain natriuretic peptide; BUN, blood urea nitrogen; CAD, coronary artery disease; CRP, C-reactive protein; H2 blocker, histamine H2-receptor antagonist; HbA1c, glycated hemoglobin; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; NGSP, National Glycohemoglobin Standardization Program; PPI, proton pump inhibitor; γ-GTP, γ-glutamyl transpeptidase; T-Cho, total cholesterol; TG, triglycerides **p* < 0.05, ***p* < 0.01.

were two-sided, and a value of *p* < 0.05 was considered statistically significant.

Data Availability

All data from this study are available from the corresponding author following reasonable requests. The sequencing data have been deposited into the DNA databank of the Japan BioProject database with links to the BioProject accession number PRJDB7456.

Results

Participant Characteristics at Baseline and Differences in the Profiles of the Gut Microbes

Table 1 presents the baseline characteristics of the study participants. Patients with CAD were significantly older than the non-CAD control individuals. The groups did not differ in terms of sex, the body mass index, and smoking history, and the groups were comparable in terms of the laboratory data, with the

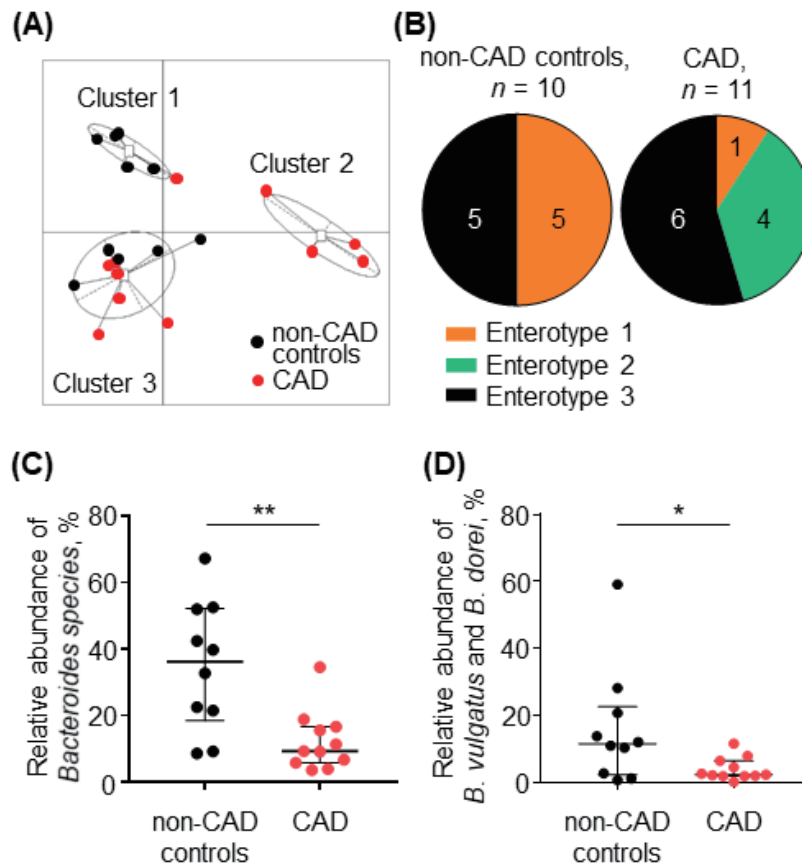


Fig. 1. Gut microbial characteristics of participants with or without coronary artery disease (CAD)

The V3–V4 regions of the bacterial 16S ribosomal ribonucleic acids in fecal samples from 10 non-CAD control subjects and 11 CAD patients were sequenced. (A) The participants were categorized into three clusters based on the abundance at the genus level in the gut microbiota. (B) Distributions of the samples from non-CAD control individuals and CAD patients in each cluster. Chi-squared test was used to compare the three clusters. (C) Relative abundance of *Bacteroides* spp. Data are presented as the medians and interquartile ranges (25th–75th percentiles). (D) Relative abundance of *Bacteroides vulgatus* and *Bacteroides dorei*. Data are presented as medians and interquartile ranges (25th–75th percentiles). * $p < 0.05$, ** $p < 0.01$.

exception of the lipid profiles. As patients with CAD were required to reduce their plasma LDL levels for secondary prevention, they all were prescribed statins. The groups did not differ in terms of the ratio of medications used for hypertension and diabetes mellitus.

To analyze the study participants' gut microbial profiles, the participant samples were grouped into three clusters at the genus level, as described previously (Fig. 1A)³⁸. Cluster 1 was characterized by a high abundance of *Bacteroides* spp., cluster 2 was characterized by a high abundance of *Prevotella* spp., and cluster 3 was characterized by a high abundance of other bacterial species. The enterotype of the samples from patients with CAD differed significantly from

that of non-CAD control individuals, and samples of patients with CAD were less likely to be categorized as enterotype 1 (Fig. 1B). The mean Firmicutes-to-Bacteroidetes ratio in the fecal samples from patients with CAD (2.823 ± 1.145) was significantly higher than that from non-CAD control individuals (1.51 ± 1.90) ($p = 0.0062$). The CAD fecal samples contained a significantly lower mean abundance of *Bacteroides* spp. ($12.40\% \pm 8.96\%$) than non-CAD fecal samples ($34.87\% \pm 19.41\%$) ($p = 0.0048$) (Fig. 1C). **Supplementary Fig. 2** illustrates the gut microbial analyses. CAD fecal samples contained a significantly lower level of *B. vulgatus* and *B. dorei* ($3.96 \pm 3.39\%$) than did non-CAD fecal samples ($16.00 \pm 17.45\%$) ($p = 0.043$) (Fig. 1D).

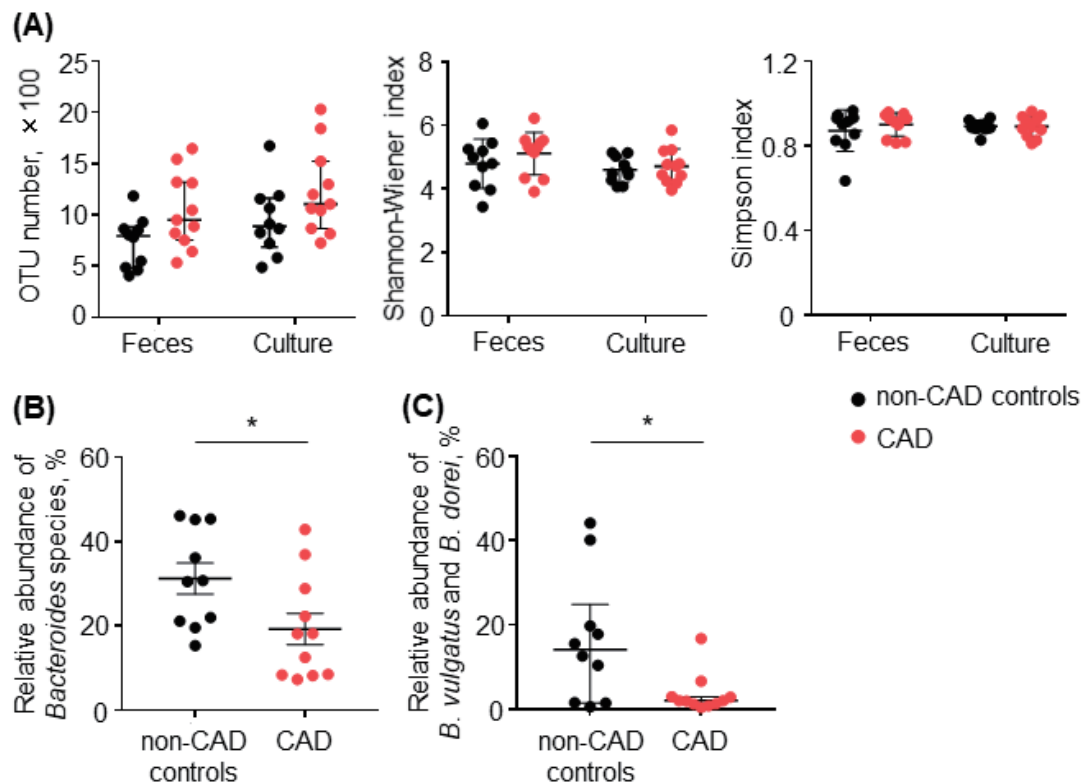


Fig. 2. Changes in the gut microbes after fermentation for 30 h

The V3–V4 regions of the bacterial 16S ribosomal ribonucleic acids in fecal sample cultures from 10 non-coronary artery disease (CAD) control subjects and 11 CAD patients were sequenced. (A) Operational taxonomic unit numbers, Shannon–Wiener index, and Simpson index. Data from original fecal samples (Feces) and cultures of samples that had been fermented for 30 h in the Kobe University Human Intestinal Microbiota Model (Culture) are presented. The data presented are the medians and interquartile ranges (25th–75th percentiles). Wilcoxon signed-rank test was used to compare the samples from the same patients. (B) Relative abundance of *Bacteroides* spp. in the cultures. The data presented are the means and standard errors of the means. (C) Relative abundance of *Bacteroides vulgatus* and *Bacteroides dorei* in the cultures. The data presented are the medians and interquartile ranges (25th–75th percentiles). * $p < 0.05$.

Microbial Compositions of the Fecal Samples and Cultures Following Fermentation

We examined whether KUHIMM could simulate the impaired gut microbiota of patients. Specifically, we compared the microbial composition of original fecal samples (feces samples) with that of samples that had been fermented for 30 h in the KUHIMM (culture samples). The numbers of OTUs, Shannon–Wiener indexes, and Simpson indexes were similar in the feces and culture samples (Fig. 2A). There were no major changes in the relative abundance of bacteria at the phylum level in CAD fecal samples or in non-CAD fecal samples (data not shown). The relative abundance of *Bacteroides* spp., *B. vulgatus*, and *B. dorei* in CAD fecal cultures were significantly lower than those in non-CAD fecal cultures (Fig. 2B, Fig. 2C).

Impact of RS on Fecal Bacterial Cultures from the Gut Microbiota

RS did not significantly alter the numbers of total bacteria in non-CAD fecal culture (without RS: $7.55 \pm 2.59 \times 10^{10}$ copies/mL; with RS: $7.96 \pm 2.10 \times 10^{10}$ copies/mL, $p = 0.66$) or in CAD fecal culture (without RS: $7.06 \pm 2.73 \times 10^{10}$ copies/mL; with RS: $8.92 \pm 4.27 \times 10^{10}$ copies/mL, $p = 0.32$). The numbers of OTUs in non-CAD control fecal cultures and CAD fecal cultures that had and had not been fermented with RS were similar (Supplemental Fig. 3A, B). There were no differences between the cultures of non-CAD or CAD fecal samples that did or did not contain RS in terms of the Shannon–Wiener index and Simpson index (Supplementary Fig. 3A, Supplementary Fig. 3B).

RS did not alter the relative abundance of *Bacteroides* in the cultures of non-CAD fecal samples

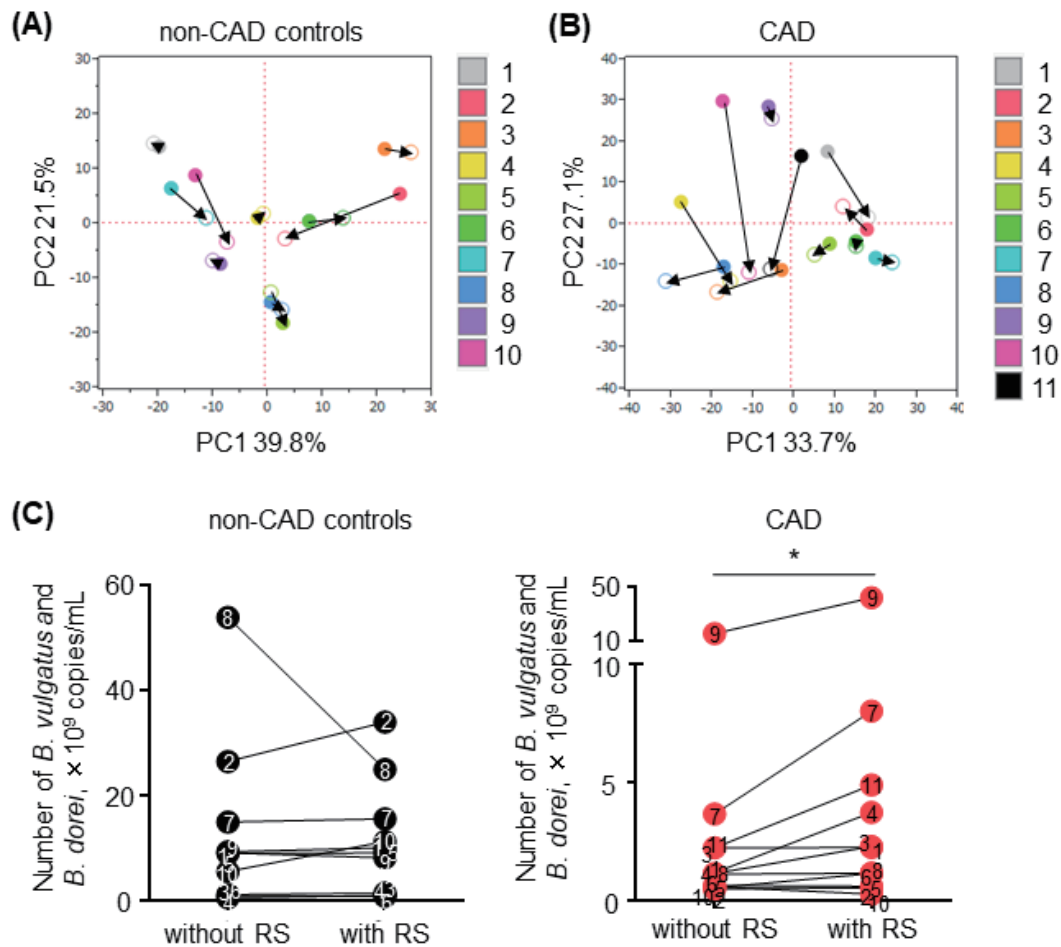


Fig. 3. Resistant starch (RS) altered the bacterial composition in the Kobe University Human Intestinal Microbiota Model

(A) Principal component analysis (PCA) score plots at genus level with (circle) and without (dot) RS in 10 non-CAD control subjects. (B) PCA score plots at genus level with (circle) and without (dot) RS in 11 CAD patients. (C) Relative increase of *Bacteroides vulgatus* and *B. dorei*.

(without RS: $33.48 \pm 11.38\%$; with RS: $32.49 \pm 9.61\%$, $p=0.60$) or CAD fecal samples (without RS: $27.27 \pm 11.61\%$; with RS: $30.61 \pm 9.22\%$, $p=0.34$). **Supplementary Fig. 4** shows the relative distributions of the gut microbes at the genus level in the cultures that did and did not contain RS. **Fig. 3A** and **Fig. 3B** present the principal component analysis score plots at the genus level for the cultures that did and did not include RS. RS supplementation did not change the abundance of *Bacteroides* in the non-CAD fecal cultures (without RS: $31.24\% \pm 11.62\%$; with RS: $28.98\% \pm 10.01\%$, $p=0.21$) or in CAD fecal cultures (without RS: $19.33\% \pm 12.3\%$; with RS: $17.73\% \pm 10.78\%$, $p=0.34$).

The quantitative PCR analysis showed that RS did not significantly change the absolute numbers of *B. vulgatus* and *B. dorei* in non-CAD fecal cultures (without RS: $1.31 \pm 1.63 \times 10^{10}$ copies/mL; with RS:

$1.07 \pm 1.08 \times 10^{10}$ copies/mL, $p=0.38$), but some cultures showed increases in the abundance of *B. vulgatus* and *B. dorei* (**Fig. 3C**). RS significantly increased the absolute numbers of *B. vulgatus* and *B. dorei* in CAD fecal cultures (without RS: $2.67 \pm 4.38 \times 10^9$ copies/mL; with RS: $6.08 \pm 1.21 \times 10^9$ copies/mL, $p=0.019$) (**Fig. 3C**). The absolute numbers of *B. vulgatus* and *B. dorei* declined in a few samples (**Fig. 3C**).

Impact of RS on SCFA Production

To elucidate the impact of RS on SCFA production, we measured the SCFA levels after fermentation with or without RS for 30 h. RS did not significantly change the acetate or propionate levels in both non-CAD and CAD fecal samples (**Fig. 4A**, **Fig. 4B**). However, the presence of RS was associated with a relative or significant increase in butyrate production in both non-CAD and CAD fecal cultures (**Fig. 4B**). The

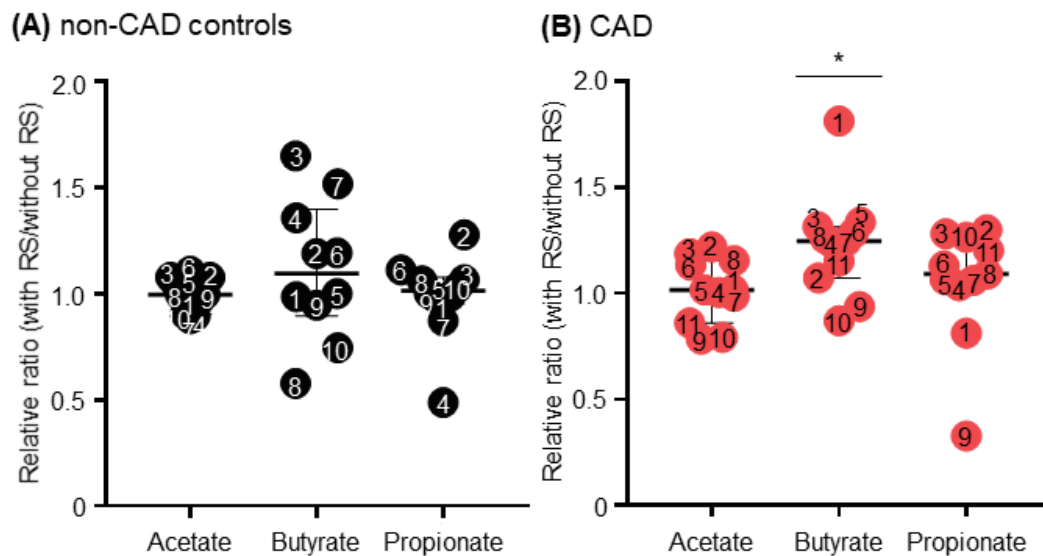


Fig. 4. Changes in short-chain fatty acid production after 30-h fermentation with resistant starch (RS) in the Kobe University Human Intestinal Microbiota Model (KUHIMM)

Fecal samples from (A) non-coronary artery disease (CAD) control subjects and (B) CAD patients were co-cultured with RS in the KUHIMM, and changes in the production of short-chain fatty acids after 30 h of fermentation were analyzed. The changes are presented as ratios relative to the without-RS (Culture) group. The data presented are the medians and interquartile ranges (25th–75th percentile). * $p < 0.05$.

SCFA production profiles differed among the individuals.

Discussion

Increasing evidence has been supporting a robust relationship between the gut microbiota and the incidence of cardiovascular diseases^{3–7}. Our previous translational research findings have shown that reductions in the abundance of *B. vulgatus* and *B. dorei* in the gut microbiota contribute to the incidence of CAD¹². These results suggest that increasing the abundance of *B. vulgatus* and *B. dorei* within the gut microbiota may prevent and treat CAD.

Several clinical studies have demonstrated the beneficial effects of dietary fiber on glycemic control, cholesterol level control, blood pressure regulation, and body weight control³⁹. Following improvements in the management of these coronary risk factors, dietary fiber can prevent the future incidence of CAD^{21–23}. Although several potential mechanisms have been proposed regarding the role of RS on gut microbiota, there have been no interventional clinical trials that have investigated its role on the gut microbiota of patients with CAD. This is the first study to investigate the effect of dietary fibers on fecal sample cultures of patients with CAD and non-CAD individuals with coronary risk factors. To overcome ethical and funding considerations, we performed this *in vitro*

study using the KUHIMM, which appears to be able to simulate the human gut microbiota both metagenomically and metabolically. RS, a naturally occurring edible dietary fiber that is present in and added to food, is reported to significantly increase the relative abundance of bacteria of the phylum *Bacteroidetes* in mice³¹. We chose RS as a supplement, and investigated its effect on the abundance of *B. vulgatus* and *B. dorei* by using quantitative PCR.

The multi vessels in the KUHIMM are smaller than other *in vitro* models⁴⁰. The KUHIMM is capable of reproducing the number and diversity of microbiota species at the same level as observed in human fecal samples^{28–30}. Although the KUHIMM can replicate a healthy human gut microbiota and maintain its diversity, we have never tried to ferment the gut microbiota of CAD patients. Our results indicated that 30-h fermentation in the KUHIMM did not lead to significant changes in the numbers of OTUs or the α -diversities in CAD and non-CAD fecal cultures. In addition, 30-h fermentation in the KUHIMM showed decreased relative abundance of *B. vulgatus* and *B. dorei* in the CAD fecal cultures compared non-CAD control fecal cultures. These results suggest that the KUHIMM could be used to simulate gut microbiota for prebiotic evaluation in patients with CAD. Contrary to our expectations, our results showed that RS did not uniformly alter the abundance of bacteria of the phylum *Bacteroidetes* and of the genus *Bacteroides*

in non-CAD and CAD fecal sample cultures in the KUHIMM. Although we found that RS could significantly increase the abundance of *B. vulgatus* and *B. dorei* in CAD fecal sample cultures, this effect varied among patients. Fecal sample analysis determined that most CAD fecal samples could be categorized as enterotypes 2 or 3 and that they had a lower abundance of *B. vulgatus* and *B. dorei*, suggesting that RS supplementation could be more beneficial for patients with CAD than for control individuals without CAD. The heterogeneous responses to RS could be explained by the complexity of the gut microbial ecosystem⁴¹. Functional differences cause the intestinal microbiota to either compete or cooperate for nutritional substrates, resulting in the presence of a dynamic microbial network. These complex networks within individuals generate different RS degradation potentials, and induce the formation of gut microbiota with different microbial profiles. Although the recommended daily adult intakes of dietary fiber range from 30 to 35 g for men and from 25 to 32 g for women⁴², the mean daily intakes of dietary fiber within the Japanese general population are about 18.8 ± 10.2 g for men 18.0 ± 8.0 g for women⁴³. The results from this study could be used to promote food education among patients with CAD who are RS responders.

We demonstrated that RS supplementation increased butyrate production in CAD fecal sample cultures, suggesting that the gut microbial metabolites could be altered by RS assimilation. SCFAs are used as energy sources by host cells and the gut microbiota, influence the physiology of the colon, help shape the gut environment, and function as signaling molecules to regulate host metabolism and immune responses⁴⁴. RS supplementation increases fecal butyrate concentrations in healthy young adults⁴⁵. Moreover, butyrate is the predominant fermentation product from RS⁴⁶. In this study, we demonstrated, for the first time, that RS could increase butyrate production in CAD fecal sample cultures in the KUHIMM. Butyrate supports the function of the mucosal barrier by stimulating intestinal mucous production, and it induces the differentiation of colonic regulatory T cells in mice²⁶. The findings from animal experiments have shown that a butyrate-supplemented diet suppresses atherosclerotic plaque formation in apolipoprotein E-knock-out mice via reductions in the expression of chemokines and adhesion molecules at the site of the lesion, which were followed by lower levels of macrophage migration⁴⁷. Furthermore, peritoneal macrophages from butyrate-treated mice exhibited lower levels of reactive oxygen species generation⁴⁸. On the contrary, bacteria of the phylum *Bacteroidetes* produce propionate⁴⁹. RS did not increase propionate production

nor did it increase the relative abundance of *Bacteroidetes* bacteria. Although few studies have shown that SCFAs protect against atherosclerosis, this evidence offers an insight into the pathophysiology of atherosclerosis as a chronic inflammatory diseases, and we could expect the development of novel therapeutic approaches that prevent CAD progression by modulating the gut microbiota through dietary fiber consumption.

The present study had several limitations, which should be considered when interpreting the results. First, this was a study with a relatively small number of Japanese patients. Large cohort studies are needed to ascertain these observations. Second, we mainly focused on the effect of RS on the changes in the abundance of *B. vulgatus* and *B. dorei*, and SCFA production in this study. Other important changes might exist, warranting future research.

Conclusion

We have, for the first time, demonstrated heterogeneous responses of the gut microbiota and SCFA production following RS supplementation of CAD and non-CAD fecal samples cultures in the KUHIMM. The KUHIMM may be useful for pre-clinical testing of the effects of RS on the cultures of gut microbiota from patients' fecal samples and their metabolites. Our results pave the way for further investigations on gut microbial response to RS supplementation and CAD prevention and management.

Conflict of Interest

The authors declare they have no conflicts of interest.

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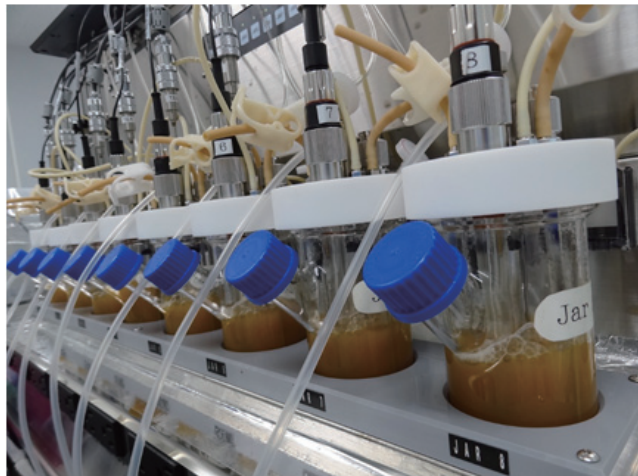
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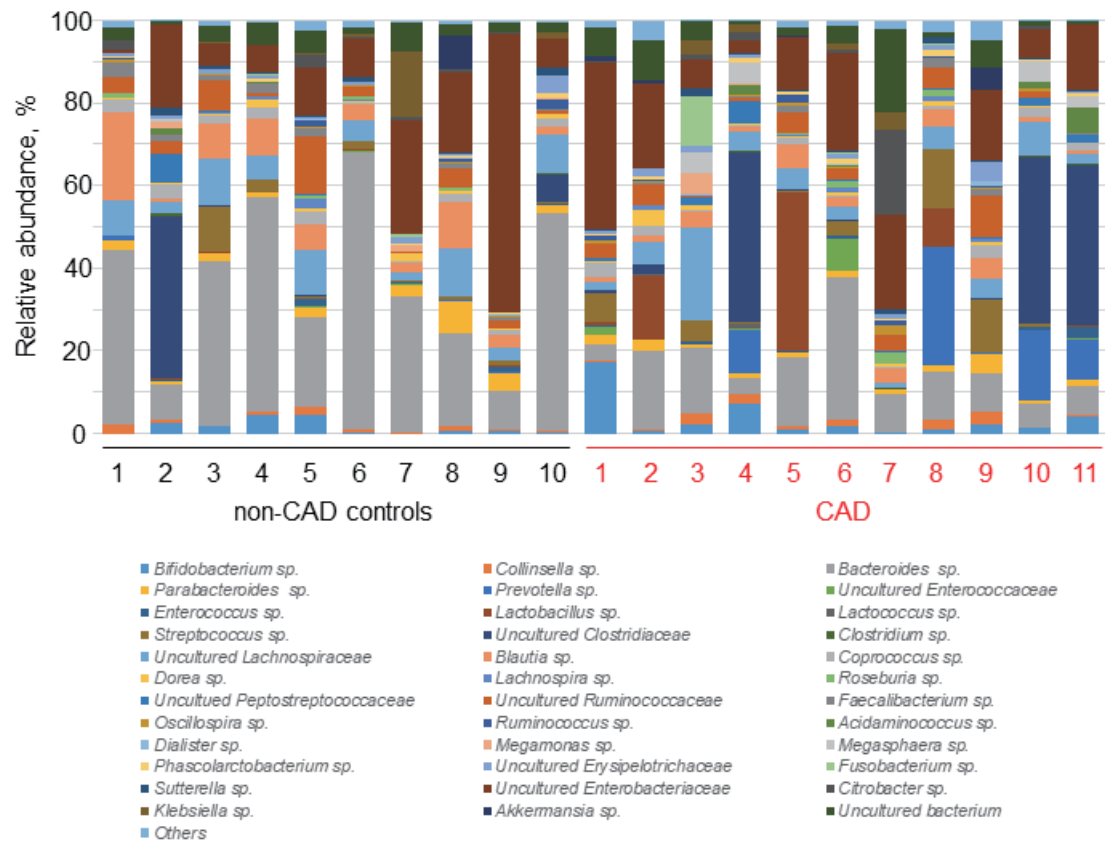
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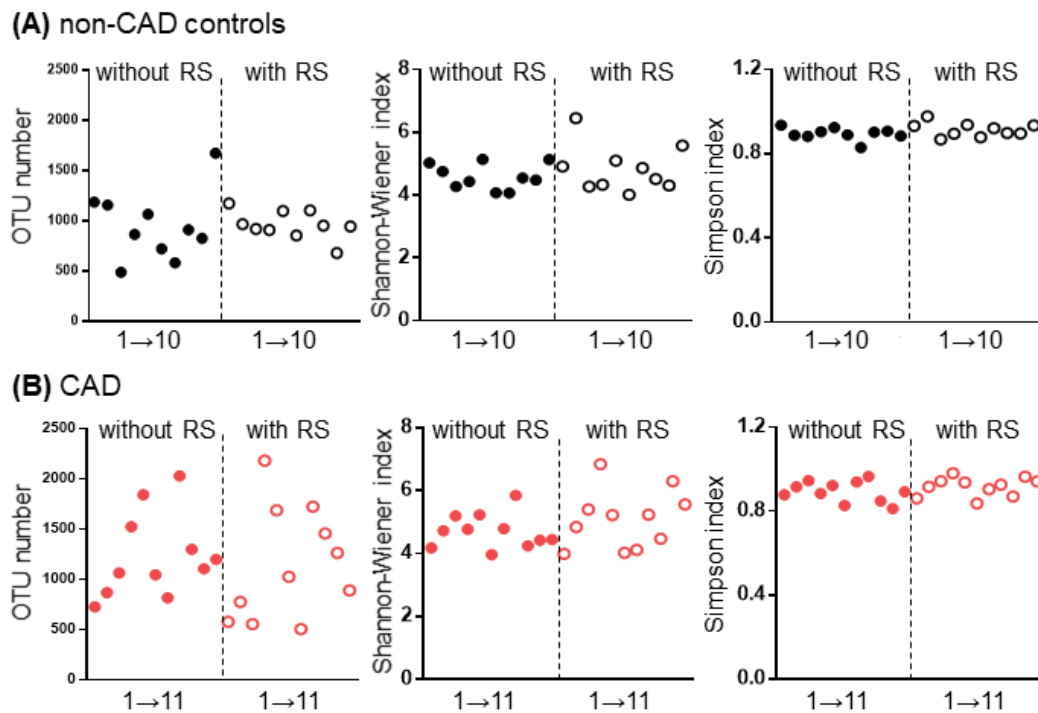


Supplementary Fig. 1. Kobe University's Human Intestinal Microbiota Model is a single-batch fermentation system composed of eight parallel and independent anaerobic culturing vessels



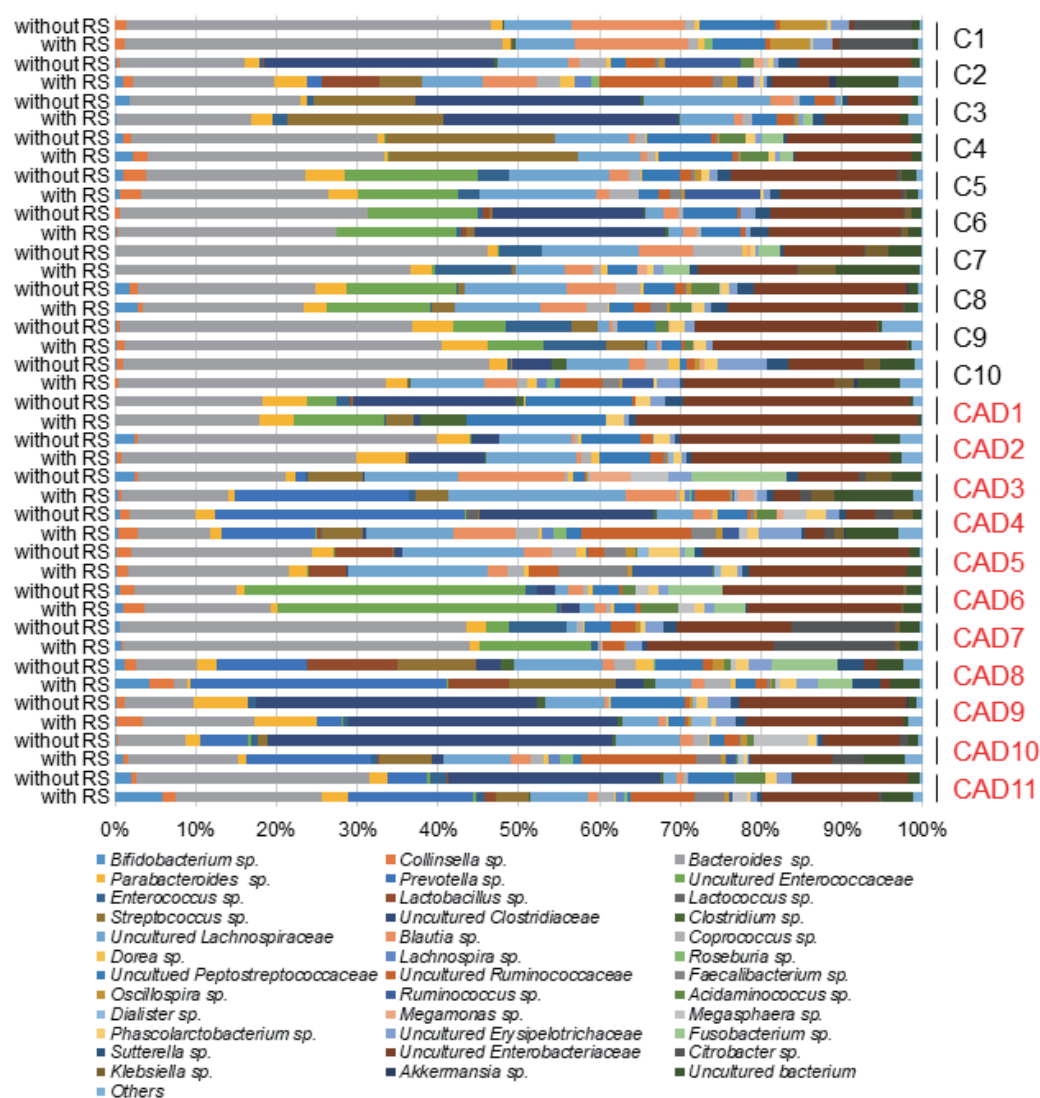
Supplementary Fig. 2. Gut microbiota of patients with or without coronary artery disease (CAD)

Feces were collected from 10 non-CAD control subjects and 11 CAD patients. The V3–V4 region of the bacterial 16S ribosomal ribonucleic acid was sequenced. The relative abundance of the microbial genera is shown. Genera with a lower abundance (<1.0%) and lower levels of similarity (<97%) were classified as Others and Uncultured bacteria, respectively.



Supplementary Fig. 3. Bacterial 16S ribosomal ribonucleic acid gene sequencing data and α -diversity values after incubation with resistant starch (RS) in Kobe University's Human Intestinal Microbiota Model

The operational taxonomic unit numbers, Shannon–Wiener index, and Simpson index for each culture incubated with or without RS are shown. (A) Non-coronary artery disease (CAD) control subjects. (B) CAD patients.



Supplementary Fig. 4. Genus-level compositional view of the bacteria after fermentation for 30 h

Samples were obtained from each vessel of Kobe University's Human Intestinal Microbiota Model (KUHIMM) after 30 h of fermentation without resistant starch (RS) and with RS. The bacterial 16S ribosomal ribonucleic acid gene sequences were analyzed, and the relative abundance at the genus level are presented. Genera with a lower abundance ($<1.0\%$) and lower levels of similarity ($<97\%$) were classified as Others and Uncultured Bacteria, respectively.