Rhamnan sulphate from green algae *Monostroma nitidum* improves constipation with gut microbiome alteration in double-blind placebo-controlled trial.

Yasuhito Shimada (shimada.yasuhito@mie-u.ac.jp)
Mie University
Masahiro Terasawa
Konan Chemical Manufacturing Co., Ltd.
Fumiyoshi Okazaki
Mie University
Hiroko Nakayama
Mie University
Liqing Zang
Mie University
Kaoru Nishiura
Konan Chemical Manufacturing Co., Ltd.
Koichi Matsuda
Konan Chemical Manufacturing Co., Ltd.
Norihiro Nishimura
Mie University

Research Article

Keywords: Seaweeds, obesity, metabolic syndrome, natural products, human study

Posted Date: April 9th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-273210/v1

Version of Record: A version of this preprint was published at Scientific Reports on July 5th, 2021. See the published version at https://doi.org/10.1038/s41598-021-92459-7.

Abstract

Rhamnan sulphate (RS), a sulphated polysaccharide from *Monostroma nitidum*, possesses several biological properties that help in treating diseases such as viral infection, thrombosis, and obesity. In the present study, we first administered RS (0.25 mg/g food volume) orally to high-fat diet-treated mice for 4 weeks. RS increased the faecal volume and calorie excretion with decreased plasma lipids, which was in accordance with the results of our previous zebrafish study. Notably, as the excretion amount by RS increased in the mice, we hypothesised that RS could decrease the chance of constipation in mice and also in human subjects because RS is considered as a dietary fibre. We administrated RS (100 mg/day) to subjects with low defaecation frequencies (3–5 times/week) for 2 weeks in double-blind placebo-controlled manner. As a result, RS administration significantly increased the frequency of dejection without any side effects, although no effect was observed on the body weight and blood lipids. Moreover, we performed 16s rRNA-seq analysis of the gut microbiota in these subjects. Metagenomics profiling using PICRUSt revealed functional alternation of the KEGG pathways, which could be involved in the therapeutic effect of RS for constipation.

Introduction

Seaweeds contain high levels of iodine, iron, vitamin C (which aids iron absorption), anti-oxidants, soluble and insoluble fibre, vitamin K, vitamin B-12, and various nutrients that promote human health. Of these, green algae belong to *Monostroma* genus, and are commercially cultivated in East Asia and South America for edible purposes, as popular sushi wraps. Rhamnan sulphate (RS) is a sulphate polysaccharide comprising L-rhamnose and sulphated L-rhamnose found in green algae, and was purified as the main constituent from the cell walls of *Monostroma lattisimum and Monostrom nitudum* in 1998[1], as an activator for anti-thrombin[2]. For the following 20 years, several biological activities of RS have been identified such as its anti-coagulant[3–6] and anti-viral effects[7–10] and others[11]. Of these, we discovered lipid-lowering properties of RS to improve hepatic steatosis, using a diet-induced obesity model of zebrafish in 2015.[12] In the present study, to validate the anti-obesity properties of RS in mammals, we administered RS to high-fat diet-induced obese mice and found that RS increased the excretion amount and calories with lipid-lowering effects. Furthermore, we performed a clinical trial on subjects with constipation tendency to determine whether RS can be used as a therapeutic molecule.

Results

Rhamnan sulphate (RS) increases faecal amount in obese mice

After 4-week feeding, RS revealed a tendency ($p < 0.1$) to suppress body weight increase in the HFD group (48.6 ± 4.4 g in HFD vs. 45.0 ± 4.6 g in HFD + RS group; Fig. 1A). Corresponding to the suppression of body weight, plasma triglycerides (49.8 ± 4.4 mg/dL in HFD vs. 36.6 ± 8.9 mg/dL in HFD + RS group;

Fig. 1B) and total cholesterol (131.9 ± 3.2 mg/dL in HFD vs. 116.1 ± 14.6 mg/dL in HFD + RS group; Fig. 1C) were significantly ($p < 0.05$) suppressed by RS. Moreover, RS significantly ($p < 0.05$) suppressed fasting blood glucose (129.8 ± 28.7 mg/dL in HFD vs. 101.8 ± 14.4 mg/dL in the HFD + RS group; Fig. 1D). For faecal analysis, RS significantly ($p < 0.05$) increased the faecal weight (0.75 ± 0.12 g/day in HFD vs. 0.90 ± 0.15 g/day in HFD + RS group; Fig. 1E) and calories (13.3 ± 0.6 kcal/day in HFD vs. 15.9 ± 2.2 kcal/day in HFD + RS group; Fig. 1F) in HFD groups.

RS improved constipation in subjects with low defaecation frequency

From the mouse experiment, we hypothesised that RS has therapeutic properties to improve constipation, thereby subsequently decreasing blood lipids and body weight. Thus, we performed a clinical trial with chronic constipation. The present study was performed from 28th February to 23rd April 2020. Seventy-three volunteers were initially screened, as illustrated in **Figure S2**. The final 38 healthy volunteers (participants), who had relatively low defaecation frequencies (3–5 times a week), were randomly allocated to the groups to receive RS or placebo. The baseline characteristics of the participants are summarized in **Table 1**. No significant difference was observed between RS and the placebo group for any baseline characteristics ($p \geq 0.05$). In contrast to the mouse experiment, RS did not decrease the body weight (Fig. 2A), plasma TG (Fig. 2B) and TCHO (Fig. 2C); however, blood glucose was significantly ($p < 0.01$) decreased by RS administration (90.7 ± 8.3 mg/dL at 0 week vs. 86.2 ± 6.0 mg/dL at 2 weeks in the RS group; Fig. 2D), which was in accordance with that of the results with mouse experiment (Fig. 1D). RS significantly ($p < 0.05$) increased the excretion frequency (4.1 ± 1.5 time/week at 0 week vs. 5.6 ± 1.9 time/week at 2 weeks in RS group; Fig. 2E) with increase in the excretion frequency from 0 to 2 weeks (0.3 ± 1.7 time/week in placebo group vs. 1.5 ± 1.6 time/week in RS group at 2 weeks; Fig. 2F). Moreover, the excretion days per week were also increased by RS administration (3.8 ± 1.0 days/week at 0 week vs. 4.9 ± 1.2 days/week at 2 weeks in the RS group; **Fig. S3**). Presumably, these results are similar to those observed in our mouse study (Fig. 1E and 1F). No important harms or unintended effects was observed during the study.

RS alters microbiota composition in subjects

At the phylum level, *Firmicutes* revealed a decreasing tendency due to RS ($p = 0.06$) and placebo ($p = 0.11$; Fig. 3A), whereas *Bacteroidetes* significantly ($p < 0.05$) increased in both RS (46.2% ± 10.0% at 0 week vs. 51.0% ± 7.2% at 2 weeks) and placebo groups (43.1% ± 10.7% in 0 week vs. 48.7% ± 6.9% at 2 weeks; Fig. 3B). In the *Firmicutes* phylum, RS significantly ($p < 0.05$) decreased the proportion of *Clostridia* (36.1% ± 13.0% at 0 week vs. 31.2% ± 12.6% at 2 weeks in the RS group; Fig. 3C) and increased *Negativicutes* classes (4.6% ± 5.0% at 0 week vs. 7.5% ± 5.0% at 2 weeks in the RS group; Fig. 3D). Moreover, *Clostridiales* order was dominant in *the Clostridia* class, and revealed similar result as *the Clostridia* class. In *the Negativicutes* class, we detected four orders, and two of these, *Acidaminococcales* (1.9% ± 3.0% at 0 week vs. 3.2% ± 3.6% at 2 weeks in the RS group; Fig. 3E) and *Veillonellales* (1.7% ±

2.3% at 0 week vs. 2.7% ± 2.5% at 2 weeks in the RS group; Fig. 3F), were significantly increased by RS. At the family level, *Mogibacterium*, *Acidaminococcaceae* and *Veillonellaceae* were significantly ($p < 0.05$) decreased by RS. Interestingly, several bacteria (*Bifidobacteriaceae*, *Bacteroidaceae*, *Odoribacteraceae* and *Sutterellaceae*) were significantly ($p < 0.05$) altered in the placebo group, which were not altered in RS (**Table 2**).

Metabolic functional pathways in RS administrated subjects

To understand the RS-induced functional alterations in the gut microbiota of participants, bacterial metagenomes were predicted by PICRUSt using 16S rRNA sequencing, as previously reported[13]. Predicted proteins in each bacterium were classified into KEGG ortholog (KO) entities, which resulted in the identification of 6188 entities across all samples. Of these, 588 KOs and 553 KOs were up-regulated (> 2.0) and down-regulated (0.5<) in the RS group, whereas 593 KOs and 1425 KOs were up-regulated and down-regulated in the placebo group. The up-regulated 414 KOs and the down-regulated 130 KOs were selective in RS groups (Fig. 4A). Thereafter, we mapped the differentially expressed KOs to the KEGG Mapper in order to identify the altered pathways in the participants. As illustrated in Fig. 4B, we identified 199 and 214 pathways in the RS and placebo groups, respectively. Furthermore, 175 pathways were common in both groups and 24 pathways were specific to RS administration. Because the RS-selective pathways (**Table S4**) contained few KOs (maximum 3 KOs in lysin biosynthesis [map00300]), we further performed different analyses. We calculated the ratio of KO counts in each KEGG pathway in the common 175 pathways and found that 56 pathways were altered in the RS group compared to placebo (> 2 or < 0.5; **Table 3**). After evaluating these 56 pathways manually (represented as images in supplementary materials), we selected some representative KEGG pathways altered by RS: ‘Metabolism of xenobiotics by cytochrome p450 (map00980; Fig. 4C)’, ‘Cationic anti-microbial peptide (CAMP) resistance (ko01503; Fig. 4E)’ and ‘Nicotinate and nicotinamide metabolism (map00760; Fig. 4C)’.

Discussion

RS improved constipation

Although we identified the therapeutic effects of RS in an HFD-induced obesity model with increased defaecation, we evaluated these effects only in constipated human subjects, and not in obese populations, as it was difficult to find obese people with constipation. Even with this limitation, we confirmed that RS increased the excretion amount both in mouse and human for the first time. In general, intake of seaweeds is beneficial for gut health and improves constipation, as they contain fibres and polysaccharides. In particular, sulphated polysaccharides from marine seaweeds affect the human microbiome[14] and improve loperamide-induced constipation in rats[15]. Since RS is categorised in sulphated polysaccharides, our results seem reasonable for ameliorating constipation with alteration of gut microbiota.

Although RS significantly improved constipation-related phenotypes between 0 and 2-week administration, no significant difference was found between the RS and placebo groups at 2 weeks (Fig. 2E and 2F). As Mancabelli *et al.* previously reported that constipated people increased the diversity of gut microbiota[16], we separated the participants into two groups: those who possessed greater diversity in gut microbiota than the average diversity of whole participants (high diversity subjects) and smaller ones (less diverse subjects). Moreover, as illustrated in **Figure S4**, RS significantly ($p < 0.05$) improved constipation between placebo and RS group on excretion day per week (3.7 ± 1.6 time/week vs. 5.1 ± 1.1 time/week at 2 weeks), increased the excretion frequency (0.0 ± 2.1 time/week vs. 2.0 ± 1.7 time/week at 2 weeks), and increased the excretion days (− 0.2 ± 1.8 time/week vs. 1.4 ± 1.3 time/week at 2 weeks) in high diversity group, which are not detected in low diversity group. For the Bristol scale (faeces property), the placebo group increased ($p < 0.01$) whereas RS did not, even in the high diversity group (data not shown). An increase in the Bristol scale indicates softening of faeces. Since RS retains water as a soluble fibre in faeces, this result seems inconsistent. We hypothesised that RS-induced defaecation occurred at a faster pace than water retention by RS, and that no increase was observed in the faecal water content as well as in the Bristol scale.

RS effects on gut microbiota in various subjects

Surprisingly, the compositions of gut microbiota and KOs in the placebo group were altered more than those in the RS group (**Table 2 and** Fig. 4). Because our trial was conducted during the first wave of the coronavirus disease 2019 (COVID-19) crisis (from February to April 2020), many participants underwent lifestyle changes such as work from home, consumed home-made meals, and avoided alcohol consumption and eating out. In this situation, we identified several gut bacteria that are involved in the therapeutic effect of RS. Although *Clostridiales* and *Negativicutes* were neighbouring in *the Firmicutes* phylum, *Clostridiales* decreased (Fig. 3C) and *Negativicutes* increased (Fig. 3D) in the RS group. *Clostridia* produce medium-length fatty acids that increase water absorption, and subsequently dry up faeces, causing constipation[17]. Thus, one of the therapeutic mechanisms of RS against constipation is the decrease in *the Clostridiales* class. In particular, prebiotic supplementation in constipation patients reduced the composition of *Clostridiales*[18]. RS selectively increased *Negativicutes* (Fig. 3D) and *Acidaminococcales* (Fig. 3E). Their biological effects on constipation remain unclear; however, several studies reported that the increase in these bacteria is positively related to improved constipation. *Negativicutes* were increased by Psyllium Husk (derived from seeds of *Plantago ovata*) administration[19] and *Bifidobacterium*-based probiotic treatment[20] during constipation improvement. *Acidaminococcus* may be one of the main factors in curing constipation during faecal microbial transplantation in clinical application[21] and administration of partially hydrolyzed guar gum (water-soluble fibres) in children[22]. Moreover, *Veillonellales*, a pro-inflammatory and lactate-fermenting bacterium increased in the irritable bowel syndrome patients[23], in both RS and placebo groups (Fig. 3F), thereby revealing stress from the new lifestyle due to COVID-19 crisis.

Predictive functional analysis of gut microbiota in subjects

Combination analysis using PICRUSt and KEGG Mapper was a powerful tool to predict functional alterations in gut microbiota[24]. KOs predicted by PICRUSt revealed that the number of down-regulated KOs in the placebo group was larger than that in the RS group (Fig. 4A). We mapped these KOs to the KEGG Mapper in order to predict functional pathways differentially expressed between the RS and placebo groups (Table 3 and supplementary materials). Of these, several KOs in the pathway "metabolism of xenobiotics by cytochrome p450" were up-regulated in the RS group, whereas they were down-regulated in the placebo group (Fig. 4C). Reactions catalysed by cytochrome p450 usually turn xenobiotics, such as polysaccharides RS, to excretable metabolites[25]. This result was in accordance with that obtained in the present study. As illustrated in Fig. 4D, in "cationic anti-microbial peptide (CAMPs) resistance" pathway, several KOs were up-regulated in RS group, whereas they were down-regulated in the placebo. CAMPs are critical frontline contributors to host defence against invasive bacterial infections. For successful survival and colonisation of the host, bacteria have a series of mechanisms that interfere with CAMP activity[26]. This result suggests that RS induced CAMP resistance, which promotes the proliferation of pathogenic bacteria; however, Assoni *et al*. reported that CAMP resistance mediates the recovery of prominent gut commensals during inflammation[27]. In general, constipation is accompanied by inflammation in the gut mucosa, implying that RS would improve not only constipation but also mucosal damage by altering the composition of gut microbiota. Nicotinamide metabolism was down-regulated in the placebo group but up-regulated in the RS group (Fig. 4E). Nicotinamide, known as vitamin B3, is essential for life as it is part of the coenzyme NADH/NAD+, which is crucial for biological redox reactions. Moreover, it ameliorates experimental colitis in mice by improving host defence and enhancing bacterial clearance in *Citrobacter rodentium*-induced colitis[28] and *Staphylococcus aureus* infection in mice [29,30]. Furthermore, NAD replenishment ameliorates constipation in aged mice[31], suggesting that one of the therapeutic mechanisms of RS is the increase in bacteria related to nicotinamide synthesis and/or secretion. In particular, our functional analysis based on PICRUSt and KEGG Mapper is just a prediction; further studies are necessary to demonstrate our speculation.

Conclusion

RS promoted defaecation in mice and human subjects without any side effects and improved gut microbiota. Therefore, co-administration of RS with other prebiotics and probiotics may enhance this effect in future studies. In conclusion, with other health-promoting properties of RS (lipid-lowering, anti-viral and anti-thrombotic), it is a powerful constituent in *M. nitidum*, and can be used as a therapeutic or preventive supplement due to its anti-constipation properties.

Methods

Preparation of rhamnan sulphate (RS)

For mouse experiments, we used purified RS (> 95% purity) as previously described[7]. For the clinical study, Rhamnox® (Konan Chemical Manufacturing, Mie, Japan) was used for RS. The preparation of

Rhamnox is described as follows according to a previous study[9], with certain modifications. Dried *M. nitidum* (700 g) was washed with H_2O, by adding up to 18 L of H_2O, and then extracted for 6 h at 100°C. To prevent foaming, 2–5 g of citrate acid was added after 40 min of heating. Thereafter, celite (540 g) was added to the extract, and then centrifuged to remove the algal fronds. Next, celite (90 g) was re-added to the extract and filtered with qualitative filter paper grade 2 (Pellicon Cassette System P2B010V01; Millipore, Billerica, MA, USA). Hot water extract obtained in this manner was fractionated via ultrafiltration (Millipore; cutoff MW 10,000). The extract was sterilised at 85–90°C for 15 min and then dried using a spray drier (Ohkawara Kakohki, Kanagawa, Japan) to yield ~ 200 g of the extract. The RS content determined by gel permeation chromatography using Shodex OHpac SB-G 6B and SB-803HQ columns (eluent: 0.1M KCl, 0.15M KH_2PO_4 K_2HPO_4 buffer pH6.7) was 87.3% compared to the reference standard, which is the purified RS, as previously described[7].

Ethics

All animal procedures were approved by the Ethics Committee of Mie University and were performed according to the Japanese Animal Welfare Regulation 'Act on Welfare and Management of Animals' (Ministry of Environment of Japan) and complied with international guidelines. The clinical study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Institutional Review Board of Chiyoda Paramedical Care Clinic (Tokyo, Japan). Written informed consent was obtained from all subjects. This study was carried out in compliance with the ARRIVE guidelines (https://arriveguidelines.org/) and was registered in the University Hospital Medical Information Network Clinical Trials Registry (UMINCTR: https://upload.umin.ac.jp/cgi-open-bin/ctr_e/ctr_view.cgi?recptno=R000045150) as Number UMIN000039591 (date of registration: 25/2/2020).

Mouse experiment

NSY/HOS mice, a type 2 diabetes mellitus strain[32], was purchased from Hoshino Laboratory Animals (Saitama, Japan) and housed on a 12-h light/dark cycle at the Institute of Laboratory Animals at Mie University. Six-month-old male mice were assigned to three groups with six mice, housed individually, and were fed either the CE-7 normal diet (ND; CLEA Japan, Tokyo, Japan), high-fat diet (Test Diet 58Y1; TestDiet, Richmond, IN, USA) or a high-fat diet (HFD) supplemented with RS (250 mg/g BW) for 4 weeks to induce obesity. The compositions of ND and HFD are described in **Table S1.** During the feeding experiment, body weight, food intake, and faecal weight were measured once per week. Mice were subjected to fasting for 14 h before blood sampling to assess the fasting blood glucose levels. Blood glucose was measured using a hand-held glucometer (Glutest Neo Super; Sanwa Kagaku, Nagoya, Japan). The plasma levels of triacylglycerol (TG), low-density lipoprotein cholesterol (LDL-C), and total cholesterol (TCHO) were measured using Wako L-type TG, Wako L-type LDL-C, and Wako L-type TCHO (Wako Pure Chemicals, Osaka, Japan) assay kits according to the manufacturer's protocol. The mice were euthanized with CO_2 gas, and then the organ samples were collected and subsequently dissected for analysis.

Caloric analysis of mouse stools

The stool samples were collected daily and stored at − 20°C. The nutritional composition (fat, protein, moisture, ash, carbohydrate and energy) was determined as described elsewhere[33] with certain modifications. The fat, protein, moisture, and ash contents were evaluated by the Folch method, Kjeldahl method, atmospheric heating drying method and by direct ashing method, respectively; moreover, the carbohydrate content was assessed by subtracting the fat, protein, moisture and ash contents from the total amount. Energy content was calculated using modified Atwater factors (4, 9 and 4 kcal/g for protein, fat, and carbohydrate, respectively).

Design of clinical study

This was a randomised, double-blind, placebo-controlled, and parallel-group study carried out in a single clinical centre (Chiyoda Paramedical Care Clinic; CPCC, Tokyo, Japan) in Japan. Mie University Graduate School of Medicine and Konan Chemical Manufacturing together prepared the study protocol. All study procedures were undertaken by a clinical research organisation (CPCC) on consignment from Konan Chemical.

Subjects

Seventy-three healthy Japanese male and female volunteers (20–65 years) were selected from the total volunteers registered in the CPCC. The study details were disclosed to the subjects before enrolment, and the investigators obtained their written informed consent. Thereafter, the subjects underwent various tests (lifestyle questionnaire, medical interview, physiological, biochemical, and haematological tests). Each of these tests was performed at the CPCC. Subjects who did not meet the exclusion criteria, but met the inclusion criteria, were enrolled in the 2-week screening. Selected subjects recorded their defaecation status (number of defaecations, date of defaecation, confirmation of how many defaecations were collected on the day of defaecation, Bristol scale (faecal properties), amount of defaecation, colour of stool, smell of stool, refreshing feeling at the time of defaecation and stomach condition (abdominal pain, swelling, gurgling, bloating, flatulence, nausea) in a diary. Health adults aged 20–65 years who had relatively low defaecation frequencies (3–5 times/week) were selected for this study. There were 15 exclusion criteria, as described in **Table S2**. Eventually, 38 subjects were selected as the study subjects.

Randomization

Subjects were randomly allocated into the RS or placebo group to balance the sex, age, faecal frequency and BSS. Allocation was operated by a researcher of the CPCC who was not involved in taking measurements and analysis.

Blinding

In total, 100 mg of RS (Rhamnox®) was filled in a cellulose white capsule (Matsuya, Osaka, Japan). The placebo involved an empty capsule, identical in appearance and flavour, and was then provided to CPCC

with a coded name. Correspondence of the coded name and the true name of the product was disclosed to CPCC after completion of data analysis.

Study protocol

After a 3-week screening, 19 and 19 participants were allocated to the RS or placebo group, respectively. Each participant ingested 1 capsule/day for 2 weeks. After the end of the trial, no participant experienced adverse events. Participants recorded their life survey and defaecation questionnaire in diary every day during the study period. The test schedule is illustrated in **Figure S1**.

Data collection

The primary outcomes were defaecation frequency, defaecation date and Bristol Scale. The secondary outcomes were gut microbiota and faecal condition (faecal odour, colour, volume and feeling after defaecation). Faecal frequency and condition were assessed by recording the defaecation times and faecal condition every day in a diary. Analysis exclusion criteria are described in **Table S3**; however, no subject was excluded from the analysis.

Faecal sample collection and DNA extraction

Faecal samples were collected using a guanidine thiocyanate solution (Faeces Collection kit; Techno Suruga Lab, Shizuoka, Japan). After vigorous mixing, the samples were stored at 4°C for a maximum of 7 days until DNA extraction. After homogenisation with lysis solution F (Nippon Gene, Tokyo, Japan), the genomic DNA was heated at 65°C for 10 min and purified from the supernatants using the MPure Bacterial DNA Extraction Kit (MP Biomedicals, Solon, OH, USA) with MPure-12 system (MP Biomedicals). The purified DNA was quantified using Synergy LX (BioTek Instruments, Winooski, VT, USA) and QuantiFluor ® system (Promega, Madison, WI, USA).

Sequencing of 16S rRNA gene

Illumina MiSeq paired-end sequencing of the hypervariable V3–V4 regions of the 16S rRNA was performed at Bioengineering Lab. Co., Ltd. (Kanagawa, Japan). A two-step, tailed PCR approach was used according to the protocol for 16S metagenomic sequencing library preparation (Illumina, San Diego, CA, USA). Both the V3 and V4 regions of the 16S ribosomal RNA were amplified with primers containing the Illumina overhang adaptor (forward primer 5' ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT CCT ACG GGN GGC WGC AG; Reverse primer 5' GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC TGA CTA CHV GGG TAT CTA ATC C). Thereafter, Index PCR was performed with Index 1 and Index 2 primers from the Nextera XT Index Kit (Illumina), using 2 μL of amplicon derived from the previous PCR. Next, the indexed libraries were cleaned and analysed with a Fragment Analyser, using a dsDNA 915 Reagent Kit (Advanced Analytical Technologies, Ames, IA, USA). The prepared libraries were used for paired-end sequencing using MiSeq v3 reagents and 2 × 300-bp reads on the MiSeq (Illumina).

Analysis of bacterial composition in 16S rRNA datasets

The paired-end reads of the 16S rRNA gene were assembled using QIIME2 (ver. 2020.2), with the default parameter values, were applied for sequence de-noising, primer sequence trimming and chimaera checking using the DADA2 method[34,35]. Quality filtered reads were assigned to operational taxonomic units (OTUs) (100% identity) using *de novo* OTU picking and taxonomic assignment using the feature-classifier plug-inn against the EzBioCloud 16S database (https://www.ezbiocloud.net/)[36].

Microbiome analysis

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2, version 2.3.0b)[37] was used to predict the functional gene content in the faecal microbiota based on the taxonomy obtained from the EzBioCloud 16S database. Subsequently, the acquired KEGG orthologs were mapped using the KEGG Mapper[38] as previously reported[24].

Safety assessment

The principal investigator assessed the safety of RS based on the results of participant communication, tests (physiological, biochemical, haematological) and urinalyses. The daily diary content was also referred to for safety assessment.

Sample size

Although RS was tested on subjects with constipation, no testing was performed on these healthy subjects; hence, we were unable to estimate the minimum number of subjects. Thereafter, we set the minimum number of subjects to 19 for statistical analysis.

Statistical analysis

All results were represented as means with standard deviations (SD). Data were analysed using Student's t-test or one-way ANOVA with the Dennett multiple comparison procedure, depending on the number of comparisons, using GraphPad Prism version 7 (GraphPad Software, San Diego, CA, USA) or IBM SPSS software (IBM, Armonk, NY, USA).

Declarations

Acknowledgements

We thank Ms. Takako Taguchi and Ms. Azusa Kato for their secretarial assistance. We would like to thank Editage (www.editage.jp) for English language editing. Financial support for this study was provided by Konan Chemical Manufacturing Co., Ltd.

Authors' contributions

Conceptualisation, Y.S., M.T., and N.N.; Methodology—Chemistry, K.N. and K.M.; Methodology—mouse study, Y.S., H.N., and L.Z.; Formal Analysis, M.T., Y.S. and F.O.; Investigation, F.O.; Resources, K.N., and

K.M.; Writing—Original Draft Preparation, Y.S. and M.T.; Writing—Review & Editing, Y.S. and M.T.; Project Administration, N.N.; Funding Acquisition, N.N.

Competing Interest Statement

Norihiro Nishimura is a board member of Konan Chemical Manufacturing Co., Ltd.; Yasuhito Shimada, Liqing Zang and Norihiro Nishimura received research grants from Konan Chemical Manufacturing Co., Ltd., Tsuji Oil Mills Co., Ltd. and Rohto Pharmaceutical Co., Ltd.; Yasuhito Shimada received research grants Kawada Feather Co., Ltd. and Bankyo Pharmaceutical Co., Ltd. Masahito Terasawa, Koru Nishimura and Koichi Maeda are employees of Konan Chemical Manufacturing Co., Ltd., a chemical company. Other authors declare no conflict of interest directly relevant to the content of this manuscript.

References

1. Lee, J. B., Yamagaki, T., Maeda, M. & Nakanishi, H. Rhamnan sulfate from cell walls of Monostroma latissimum. *Phytochemistry.* **48**, 921–925 https://doi.org/10.1016/s0031-9422(97)00927-8 (1998).
2. Harada, N. & Maeda, M. Chemical structure of antithrombin-active rhamnan sulfate from Monostrom nitidum. *Bioscience Biotechnology and Biochemistry.* **62**, 1647–1652 https://doi.org/10.1271/bbb.62.1647 (1998).
3. Liu, X. *et al.* Structural Characteristics and Anticoagulant Property In Vitro and In Vivo of a Seaweed Sulfated Rhamnan. *Mar. Drugs.* **16**, https://doi.org/10.3390/md16070243 (2018).
4. Li, H. Y. *et al.* Preparation, structure and anticoagulant activity of a low molecular weight fraction produced by mild acid hydrolysis of sulfated rhamnan from Monostroma latissimum. *Bioresour. Technol.* **114**, 414–418 https://doi.org/10.1016/j.biortech.2012.03.025 (2012).
5. Li, H. Y. *et al.* Structural characterization of an anticoagulant-active sulfated polysaccharide isolated from green alga Monostroma latissimum. *Carbohydr. Polym.* **85**, 394–400 https://doi.org/10.1016/j.carbpol.2011.02.042 (2011).
6. Okamoto, T., Akita, N., Terasawa, M., Hayashi, T. & Suzuki, K. Rhamnan sulfate extracted from Monostroma nitidum attenuates blood coagulation and inflammation of vascular endothelial cells. *Journal of Natural Medicines.* **73**, 614–619 https://doi.org/10.1007/s11418-019-01289-5 (2019).
7. Terasawa, M. *et al.* Anti-Influenza A Virus Activity of Rhamnan Sulfate from Green Algae Monostroma nitidum in Mice with Normal and Compromised Immunity. *Mar. Drugs.* **18**, https://doi.org/10.3390/md18050254 (2020).
8. Wang, S. Y. *et al.* Antiviral activity against enterovirus 71 of sulfated rhamnan isolated from the green alga Monostroma latissimum. *Carbohydr. Polym.* **200**, 43–53 https://doi.org/10.1016/j.carbpol.2018.07.067 (2018).
9. Lee, J. B., Koizumi, S., Hayashi, K. & Hayashi, T. Structure of rhamnan sulfate from the green alga Monostroma nitidum and its anti-herpetic effect. *Carbohydr. Polym.* **81**, 572–577 https://doi.org/10.1016/j.carbpol.2010.03.014 (2010).

10. Lee, J. B., Hayashi, K., Hayashi, T., Sankawa, U. & Maeda, M. Antiviral activities against HSV-1, HCMV, and HIV-1 of rhamnan sulfate from Monostroma latissimum. *Planta Med.* **65**, 439–441 https://doi.org/10.1055/s-2006-960804 (1999).
11. Suzuki, K. & Terasawa, M. Biological Activities of Rhamnan Sulfate Extract from the Green Algae Monostroma nitidum (Hitoegusa). *Mar. Drugs.* **18**, https://doi.org/10.3390/md18040228 (2020).
12. Zang, L., Shimada, Y., Tanaka, T. & Nishimura, N. Rhamnan sulphate from Monostroma nitidum attenuates hepatic steatosis by suppressing lipogenesis in a diet-induced obesity zebrafish model. *Journal of Functional Foods.* **17**, 364–370 https://doi.org/10.1016/j.jff.2015.05.041 (2015).
13. Mukherjee, A. *et al.* Bioinformatic Approaches Including Predictive Metagenomic Profiling Reveal Characteristics of Bacterial Response to Petroleum Hydrocarbon Contamination in Diverse Environments. *Sci Rep.* **7**, 1108 https://doi.org/10.1038/s41598-017-01126-3 (2017).
14. Lopez-Santamarina, A. *et al.* Potential Use of Marine Seaweeds as Prebiotics: A Review. *Molecules.* **25**, https://doi.org/10.3390/molecules25041004 (2020).
15. Shimotoyodome, A., Meguro, S., Hase, T., Tokimitsu, I. & Sakata, T. Sulfated polysaccharides, but not cellulose, increase colonic mucus in rats with loperamide-induced constipation. *Digestive Diseases and Sciences.* **46**, 1482–1489 https://doi.org/10.1023/a:1010644021888 (2001).
16. Mancabelli, L. *et al.* Unveiling the gut microbiota composition and functionality associated with constipation through metagenomic analyses. *Sci. Rep.* **7**, https://doi.org/10.1038/s41598-017-10663-w (2017).
17. Johnson, S. Clostridial constipation's broad pathology. *Med. Hypotheses.* **56**, 532–536 https://doi.org/10.1054/mehy.2000.1304 (2001).
18. Chu, J. R. *et al.* Prebiotic UG1601 mitigates constipation-related events in association with gut microbiota: A randomized placebo-controlled intervention study. *World Journal of Gastroenterology.* **25**, 6129–6144 https://doi.org/10.3748/wjg.v25.i40.6129 (2019).
19. Jalanka, J. *et al.* The Effect of Psyllium Husk on Intestinal Microbiota in Constipated Patients and Healthy Controls. *International Journal of Molecular Sciences.* **20**, https://doi.org/10.3390/ijms20020433 (2019).
20. Naumova, N. *et al.* Human Gut Microbiome Response to Short-Term Bifidobacterium-Based Probiotic Treatment. *Indian Journal of Microbiology.* **60**, 451–457 https://doi.org/10.1007/s12088-020-00888-1 (2020).
21. Tian, Y. *et al.* Potential role of fecal microbiota in patients with constipation. *Therapeutic Advances in Gastroenterology.* **13**, https://doi.org/10.1177/1756284820968423 (2020).
22. Inoue, R. *et al.* Dietary supplementation with partially hydrolyzed guar gum helps improve constipation and gut dysbiosis symptoms and behavioral irritability in children with autism spectrum disorder. *Journal of Clinical Biochemistry and Nutrition.* **64**, 217–223 https://doi.org/10.3164/jcbn.18-105 (2019).
23. Shukla, R., Ghoshal, U., Dhole, T. N. & Ghoshal, U. C. Fecal Microbiota in Patients with Irritable Bowel Syndrome Compared with Healthy Controls Using Real-Time Polymerase Chain Reaction: An

Evidence of Dysbiosis. *Digestive Diseases and Sciences.* **60**, 2953–2962 https://doi.org/10.1007/s10620-015-3607-y (2015).

24. Okazaki, F. *et al.* Microbiome Alteration in Type 2 Diabetes Mellitus Model of Zebrafish. *Sci Rep.* **9**, 867 https://doi.org/10.1038/s41598-018-37242-x (2019).
25. Anzenbacher, P. & Anzenbacherova, E. Cytochromes P450 and metabolism of xenobiotics. *Cellular and Molecular Life Sciences.* **58**, 737–747 https://doi.org/10.1007/pl00000897 (2001).
26. Joo, H. S., Fu, C. I. & Otto, M. Bacterial strategies of resistance to antimicrobial peptides. *Philosophical Transactions of the Royal Society B-Biological Sciences.* **371**, https://doi.org/10.1098/rstb.2015.0292 (2016).
27. Cullen, T. W. *et al.* Antimicrobial peptide resistance mediates resilience of prominent gut commensals during inflammation. *Science.* **347**, 170–175 https://doi.org/10.1126/science.1260580 (2015).
28. Bettenworth, D. *et al.* Nicotinamide treatment ameliorates the course of experimental colitis mediated by enhanced neutrophil-specific antibacterial clearance. *Mol. Nutr. Food Res.* **58**, 1474–1490 https://doi.org/10.1002/mnfr.201300818 (2014).
29. Kyme, P. *et al.* C/EBP epsilon mediates nicotinamide-enhanced clearance of Staphylococcus aureus in mice. *Journal of Clinical Investigation.* **122**, 3316–3329 https://doi.org/10.1172/jci62070 (2012).
30. Hsieh, C. L. *et al.* Nicotinamide Increases Intracellular NAD(+) Content to Enhance Autophagy-Mediated Group A Streptococcal Clearance in Endothelial Cells. *Frontiers in Microbiology.* **11**, https://doi.org/10.3389/fmicb.2020.00117 (2020).
31. Zhu, X. D. *et al.* Nicotinamide adenine dinucleotide replenishment rescues colon degeneration in aged mice. *Signal Transduction and Targeted Therapy.* **2**, https://doi.org/10.1038/sigtrans.2017.17 (2017).
32. Ueda, H. *et al. Diabetologia.* **38**, 503–508 https://doi.org/10.1007/s001250050312 (1995). - A NEW ANIMAL-MODEL OF SPONTANEOUS NIDDM WITH MODERATE OBESITY
33. Sugawara, S. *et al.* The 1975 Type Japanese Diet Improves Lipid Metabolic Parameters in Younger Adults: A Randomized Controlled Trial. *Journal of Oleo Science.* **67**, 599–607 https://doi.org/10.5650/jos.ess17259 (2018).
34. Bolyen, E. *et al.* Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology.* **37**, 852–857 https://doi.org/10.1038/s41587-019-0209-9 (2019).
35. Callahan, B. J. *et al.* DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods.* **13**, 581–581 https://doi.org/10.1038/nmeth.3869 (2016).
36. Yoon, S. H. *et al.* Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. *Int J Syst Evol Microbiol.* **67**, 1613–1617 https://doi.org/10.1099/ijsem.0.001755 (2017).
37. Langille, M. G. I. *et al.* Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nature Biotechnology.* **31**, 814–814 https://doi.org/10.1038/nbt.2676 (2013).

38. Kanehisa, M., Goto, S., Sato, Y., Furumichi, M. & Tanabe, M. KEGG for integration and interpretation of large-scale molecular data sets. *Nucleic Acids Res.* **40**, D109–114 https://doi.org/10.1093/nar/gkr988 (2012).

Tables

Table 1. Background characteristics in human subjects

Items	RS group	Control group	p value
Age (years)	48.6 ± 8.4	49.1 ± 7.2	0.8691
Sex (male/female)	8/11	9/10	1.0000
Height (cm)	166.51 ± 9.55	163.98 ± 5.96	0.3345
Body weight (kg)	58.89 ± 8.35	59.97 ± 10.31	0.7238
BMI (kg/m^2)	21.18 ± 1.84	22.23 ± 3 .18	0.2207
Systolic blood pressure (mmHg)	114.9 ± 10.7	118.1 ± 10.1	0.3559
Diastolic blood pressure (mmHg)	70.8 ± 8.8	75.8 ± 7.9	0.0760
Pulse rate (beats/min)	66.7 ± 7.2	70.3 ± 8.2	0.1560
Excretion frequency (times/week)	3.9 ± 0.8	3.9 ± 0.8	1.0000
RS (n = 19), Control (n = 19)			
mean ± SD			
p-value: unpaired t-test			

Due to technical limitations, Tables 2 and 3 are only available as a download in the supplemental files section

Figures

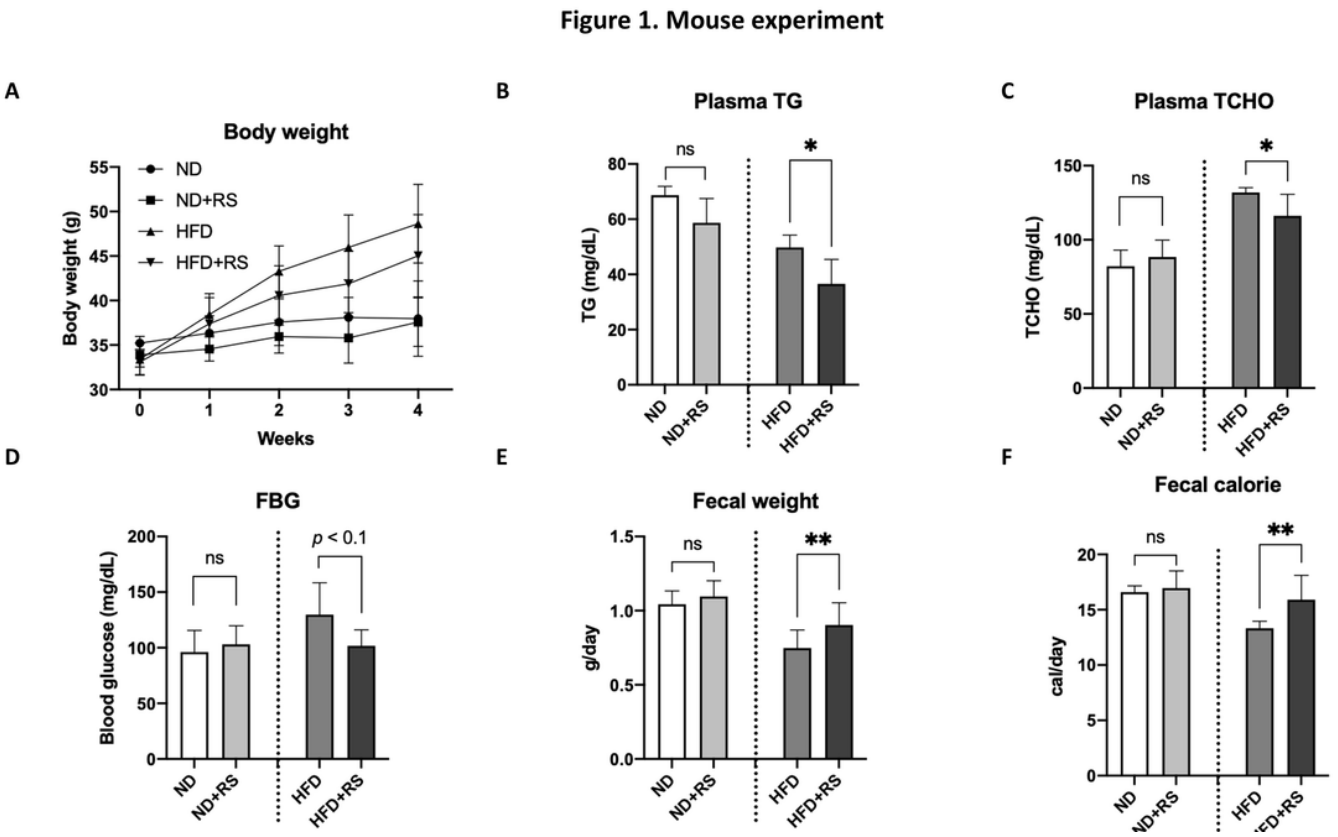

Figure 1

RS decreases blood lipids and increases faecal amount in mice with high fat diet (HFD). A. Body weight change during the feeding experiment. B–C. RS suppression increases in plasma triacylglycerol (TG; B) and total cholesterol (TCHO; C) in HFD-mice at Week 4. D. Fasting blood glucose (FBG) at Week 4. E–F. RS increased in faecal weight (E) and faecal calorie (F) in HFD-mice during the feeding experiment. *p < 0.05, **p < 0.01. n = 5, error bars indicate SD.

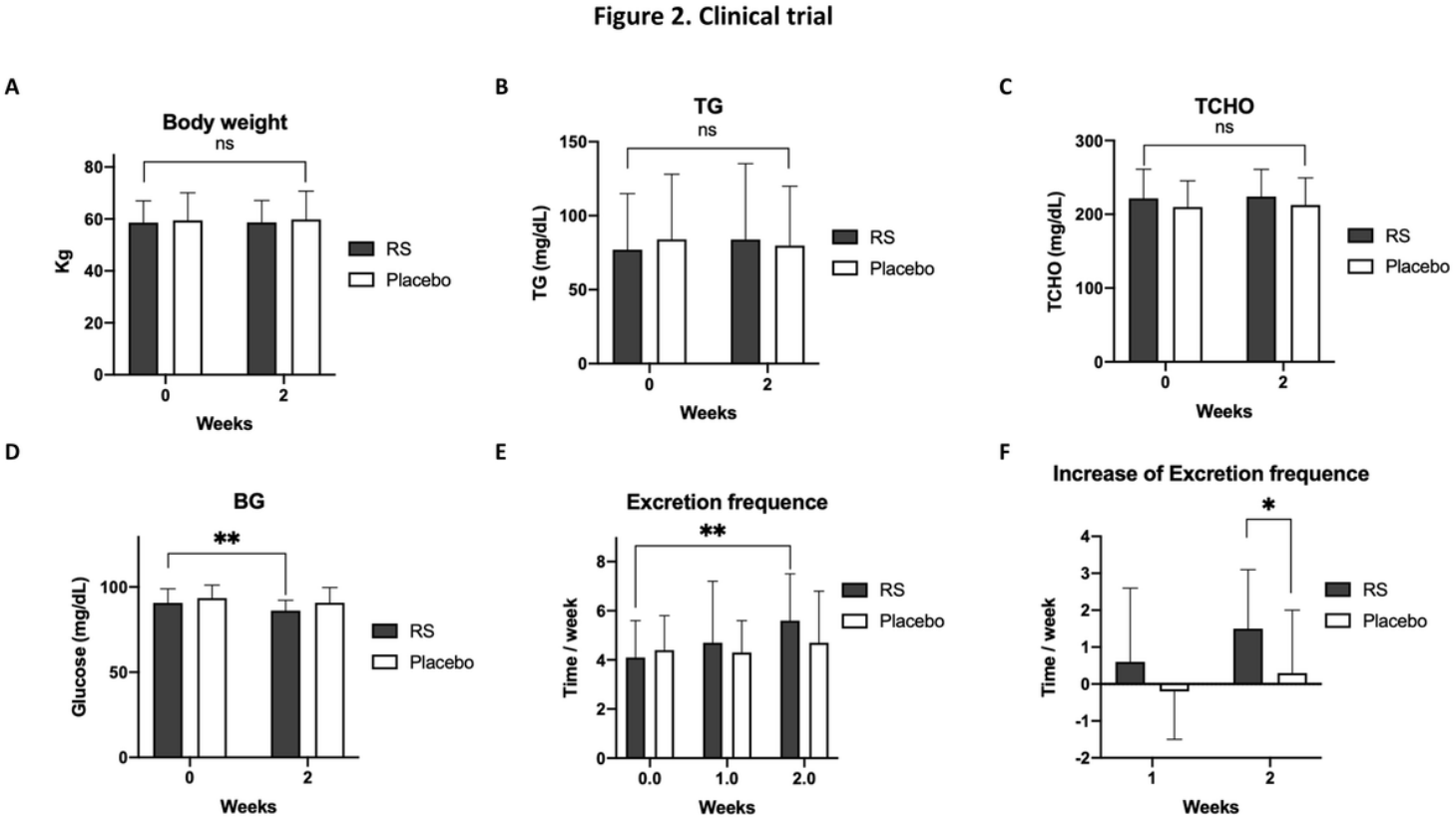

Figure 2

RS increases defaecation in human subjects. A. Body weight change during the trial. B–D. Plasma triacylglycerol (TG; B), total cholesterol (TCHO; C) and blood glucose (BG; D) at Weeks 0 and 4. E–F. RS increased in faecal frequency at Week 2. *p < 0.05, **p < 0.01. n = 19, error bars indicate SD.

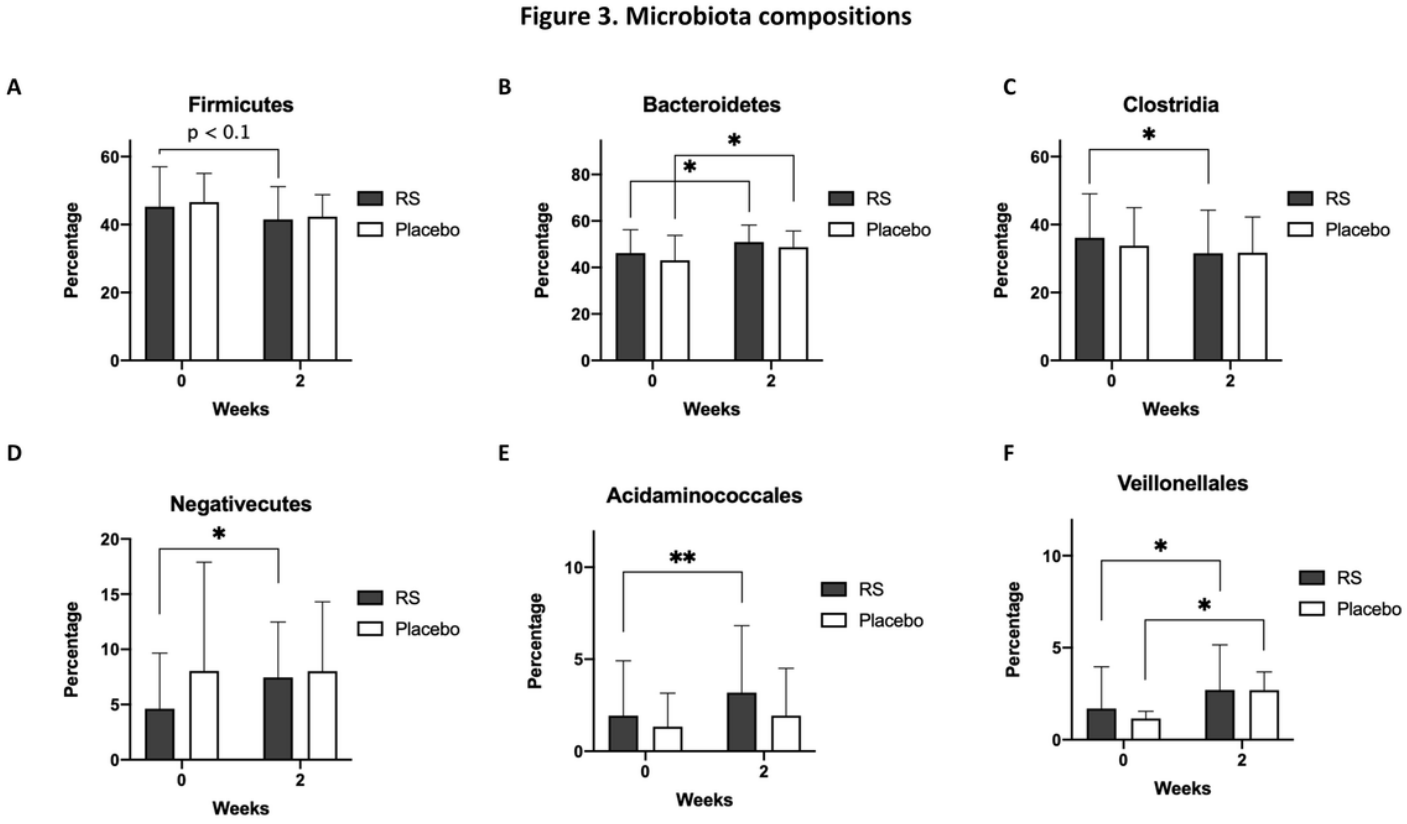

Figure 3

Difference in bacterial composition between RS and placebo group. A–B. Firmicutes (A) and Bacteroidetes (B) alteration during the trial. C–D. In Firmicutes phylum, Clostridia class decreased in RS group (C), whereas Negativicutes class increased in RS (D). E–F. In Negativicutes class, Acidaminococcales order increased in RS group (E), whereas Veilonellales increased in both groups (F). *p < 0.05, **p < 0.01. n = 19, error bars indicate SD.

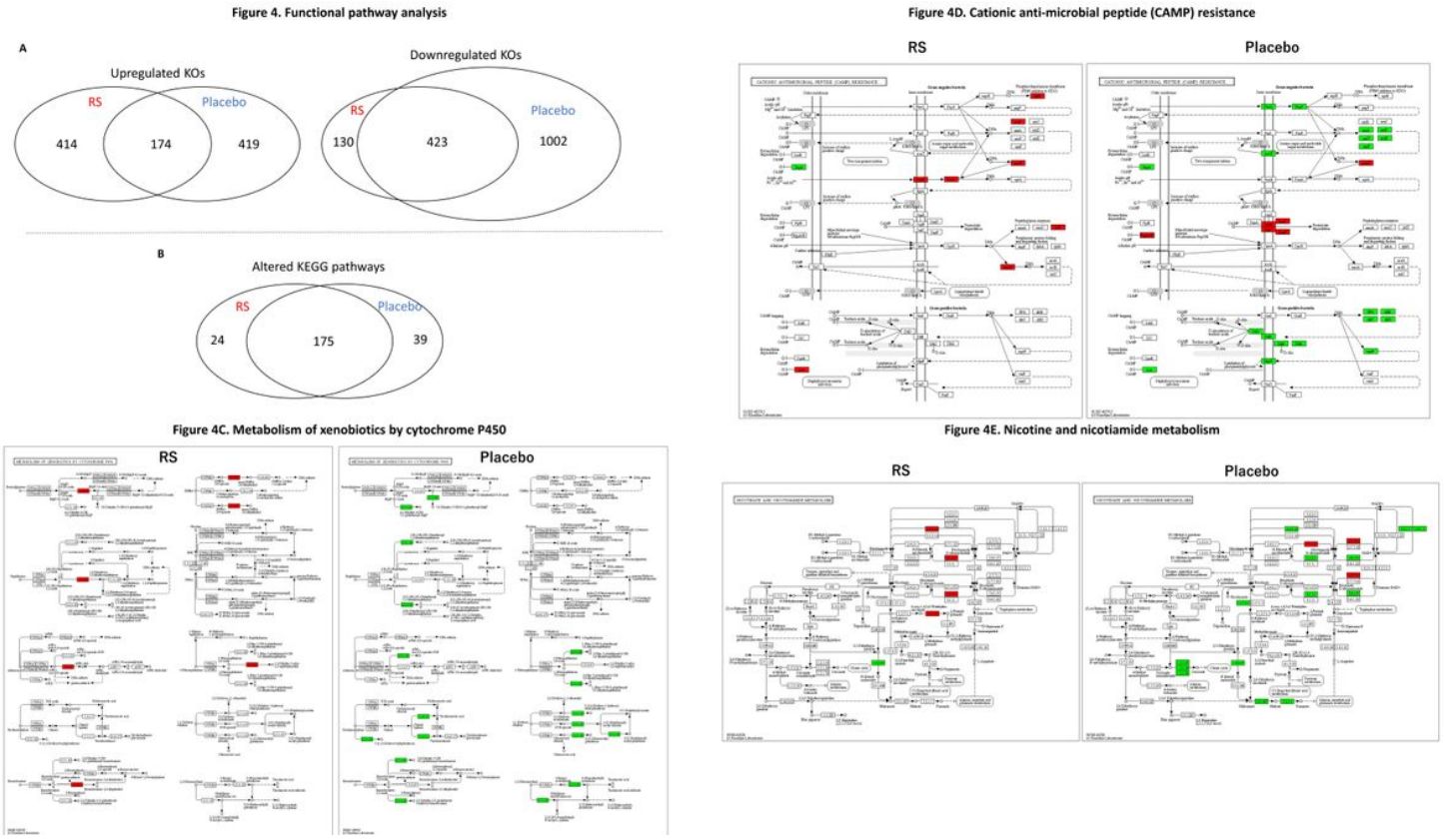

Figure 4

Alternation of microbial KEGG pathways in RS group. A. Venn diagram analysis of altered KEGG orthologues (KOs) identified PICRUSt analysis. B. Venn diagram analysis of KEGG pathways predicted by KEGG Mapper. C–E. Several KEGG pathways were up-regulated in RS group compared with those of the placebo group. Metabolism of xenobiotics by cytochrome P450 (C), cationic anti-microbial peptide (CAMP) resistance (D), and nicotine and nicotiamide metabolism (E). Red and green indicates up-regulation and down-regulation, respectively.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- 210104Table2families.xlsx
- 210104Table3Pathways.xlsx
- 210114SuppleTables.docx
- 210115supplementaryfigures.docx