



OPEN

Potential roles of 1,5-anhydro-D-fructose in modulating gut microbiome in mice

Takashi Ito^{1,2,3}✉, Takaaki Totoki^{1,3}, Seiya Takada¹, Shotaro Otsuka¹ & Ikuro Maruyama¹

The gut microbiota has tremendous potential to affect the host's health, in part by synthesizing vitamins and generating nutrients from food that is otherwise indigestible by the host. 1,5-Anhydro-D-fructose (1,5-AF) is a monosaccharide with a wide range of bioactive potentials, including anti-oxidant, anti-inflammatory, and anti-microbial effects. Based on its potential benefits and minimal toxicity, it is anticipated that 1,5-AF will be used as a dietary supplement to support general health. However, the effects of 1,5-AF on the gut microbiota are yet to be clarified. Here, using an unbiased metagenomic approach, we profiled the bacterial taxa and functional genes in the caecal microbiota of mice fed a diet containing either 2% 1,5-AF or a reference sweetener. Supplementation with 1,5-AF altered the composition of the gut microbiota, enriching the proportion of *Faecalibacterium prausnitzii*. 1,5-AF also altered the metabolomic profile of the gut microbiota, enriching genes associated with nicotinamide adenine dinucleotide biosynthesis. These findings support the potential benefits of 1,5-AF, but further studies are required to clarify the impact of 1,5-AF on health and disease.

The gut microbiota has tremendous potential to affect the host's health. It plays an important role in educating the host immune system, providing protection against pathogen overgrowth, synthesizing vitamins, and generating nutrients from food that is otherwise indigestible by the host^{1,2}. The diet and gut microbes are key determinants of the molecular composition of the host's blood³, and dysbiosis of gut microbiota is linked to a wide range of chronic disorders, including metabolic syndrome and diabetes^{4,5}.

For more than 200 million years, mammalian–microbial partnerships have adapted to local environments, in which climate, diet, and pathogens can all exert selection pressures⁶. It has become evident that environmental exposure is decisive in shaping the patterns of gut microbiota⁷. These patterns can be clustered into enterotypes, distinguished primarily by the levels of *Bacteroides* and *Prevotella*. The *Bacteroides* enterotype is associated with meat consumption, as in a Western diet, whereas the *Prevotella* enterotype is associated with a carbohydrate-based diet, as in agrarian societies or vegetarians⁸. However, the role of *Prevotella* in the host's health is controversial. While some studies suggested an association of *Prevotella* with improved glucose metabolism^{9,10}, others highlighted an association with insulin resistance and glucose intolerance¹¹. Whether *Prevotella* is beneficial or deleterious may depend on the dietary environment or the diversity existing among different strains¹². Although the causal relationships between the gut microbiota and age-associated disorders remain incompletely understood, recent studies have suggested that the strategic manipulation of the gut microbiota with prebiotics or probiotics can limit the progression of degenerative diseases in mice^{13,14}.

1,5-Anhydro-D-fructose (1,5-AF) is a bioactive monosaccharide that is formed by the degradation of glyco- gen or related molecules. It occurs in mammalian tissues, algae, fungi, and bacteria, and is metabolized in vivo to 1,5-anhydro-D-glucitol (1,5-AG)¹⁵. 1,5-AG is the second most abundant polyol in human fluids, and can be used as a marker of glycaemic control in patients with diabetes. 1,5-AF has a wide range of bioactive properties, exerting anti-oxidant, anti-inflammatory, anti-microbial, anti-diabetic, and anti-cancer effects^{15–18}. Based on its potential benefits and minimal toxicity¹⁹, it is anticipated that 1,5-AF will be used as a dietary supplement to support general health.

¹Department of Systems Biology in Thromboregulation, Kagoshima University Graduate School of Medical and Dental Science, Kagoshima, Japan. ²Department of Biomedical Laboratory Sciences, Faculty of Life Sciences, Kumamoto University, 4-24-1 Kuhonji, Kumamoto 862-0976, Japan. ³These authors contributed equally: Takashi Ito and Takaaki Totoki. ✉email: tito@kumamoto-u.ac.jp

Given the anti-microbial, anti-diabetic, and anti-oxidant properties of 1,5-AF, it can be assumed that dietary supplementation with 1,5-AF will modulate the gut microbiota, with an indirect impact on the host's health. However, to date, the influence of 1,5-AF on the gut microbiota remains unclear. Here, using an unbiased metagenomic approach, we profile the bacterial taxa and functional genes in the caecal contents of mice that consumed chow supplemented with 1,5-AF or a reference sweetener.

Methods

Study design. All experimental procedures complied with the ARRIVE guidelines and the Guideline for the Proper Conduct of Animal Experiments established by the Science Council of Japan, and were approved by the Institutional Animal Care and Use Committee of Kagoshima University, Kagoshima, Japan. Eight-week-old male C57BL/6J mice (CLEA Japan, Inc., Tokyo, Japan) were housed at room temperature (25–26 °C) under a 12-h light/dark cycle. The mice were allowed free access to water and a plant-polysaccharide-based chow (Oriental Yeast Co., Ltd, Tokyo, Japan) supplemented with either 2% 1,5-AF (SUNUS Co., Ltd, Kagoshima, Japan) or 2% erythritol as the reference supplement. The mice were randomly divided into the two groups each containing 8 animals: the 1,5-AF group and the control (erythritol) group. The sample size was estimated on the basis of preliminary unpublished data of the effects of dietary supplementation on the gut microbiota. The caecal luminal contents were harvested from the anesthetized mice at 10 weeks of age and stored in faecal collection tubes (TechnoSuruga Laboratory Co., Ltd, Shizuoka, Japan) until analysis.

DNA extraction, library preparation, and sequencing. A shotgun metagenomic analysis was performed by Takara Bio Inc. (Kusatsu, Japan). The genomic DNA was extracted from the caecal luminal contents of the mice with a NucleoSpin Soil kit (Macherey–Nagel GmbH & Co., Düren, Germany). Shotgun libraries were prepared with a ThruPLEX DNA-Seq Kit (Takara Bio Inc.) and a DNA Unique Dual Index Kit (Takara Bio Inc.), according to the manufacturer's protocols. These libraries were submitted for quality control with the Agilent 2100 BioAnalyzer (Agilent, Santa Clara, CA, USA) and 150-base pair paired-end sequencing using the Illumina NovaSeq 6000 platform (Illumina, Inc. San Diego, CA, USA).

Metagenomic analysis. The metagenomic reads were pre-processed with KneadData v0.6.1, a computational tool designed to remove low-quality reads and contaminating host sequences. Approximately 7% of the 55,555,358 ± 8,347,087 raw reads were removed as low-quality reads, and then approximately 2% were removed as host reads, yielding 50,353,995 ± 7,683,757 putative microbiome reads. These reads were then taxonomically assigned using Kaiju v1.7.2 with NCBI nr database. The relative abundances of each bacterial taxon were calculated by dividing the total number of reads assigned to a particular taxon by the total number of reads for that sample. To determine variations in microbial communities between samples, principal coordinate analysis (PCoA) was performed based on Bray–Curtis distances using the QIIME software v1.8.0 and QIIME's scripts (beta_diversity.py, principal_coordinates.py, make_emperor.py). Statistical significance of the differences between groups was calculated by PERMANOVA with 9,999 permutations using the QIIME script (compare_categories.py). Bacterial taxa that had differential abundance in the 1,5-AF group and the control group were determined by the Mann–Whitney test using the QIIME script (group_significance.py). To profile the gut microbial functions from the metagenomic sequencing data, the putative microbiome reads were mapped against the functional reference database UniRef90 using the Functional Mapping Analysis Pipeline (FMAP) v0.15 tool. Gene abundances were determined as reads per kilobase of exon per million mapped sequence reads (RPKM) and expressed as z-scores. The metagenomic analysis, including bioinformatic analysis, was performed by researchers in Takara Bio Inc. and Hokkaido System Science Co., Ltd., who were independent of the animal study.

Statistical analyses. Mann–Whitney test or White's non-parametric *t*-test²⁰ was used to compare independent groups. For multiple comparisons, P values were adjusted with the false discovery rate (FDR) algorithm²¹. Corrected P values less than 0.05 were considered significant.

Ethics approval and consent to participate. The experiments involving animals were approved by the Animal Care and Use Committee of Kagoshima University, Kagoshima, Japan.

Results

Dietary supplementation with 1,5-AF modulated the gut microbiota in mice. Among the 50,353,995 ± 7,683,757 putative microbiome reads detected in this study, 87% (43,824,256 ± 6,948,174 reads) were classified into 410 species, 124 genera, 54 families, 34 orders, 24 classes, 19 phyla, and three superkingdoms (Supplementary Material 1). The taxonomic variation across the samples was then analysed at the genus, phylum, and species levels. Principal coordinate analyses indicated that the structure of the gut microbiota in mice was distinguishable based on whether their chow was supplemented with 1,5-AF or not (Fig. 1A). This finding was consistent from the phylum level to the species level.

The proportion of *Faecalibacterium prausnitzii* was higher in mice fed with 1,5-AF diet compare with those fed with control diet. Bacteroidetes and Firmicutes are the two major phyla in the gut microbiota. The proportion of Bacteroidetes was higher in mice fed with 1,5-AF diet than those fed with control diet ($P < 0.01$). The proportion of Firmicutes was similar in both groups (Fig. 1B). In the phylum Bacteroidetes, the proportion of genus *Bacteroides* was lower ($P < 0.01$), whereas the proportion of genus *Alistipes* was higher ($P < 0.01$) in mice fed with 1,5-AF diet compare with those fed with control diet. At the species level, the propor-

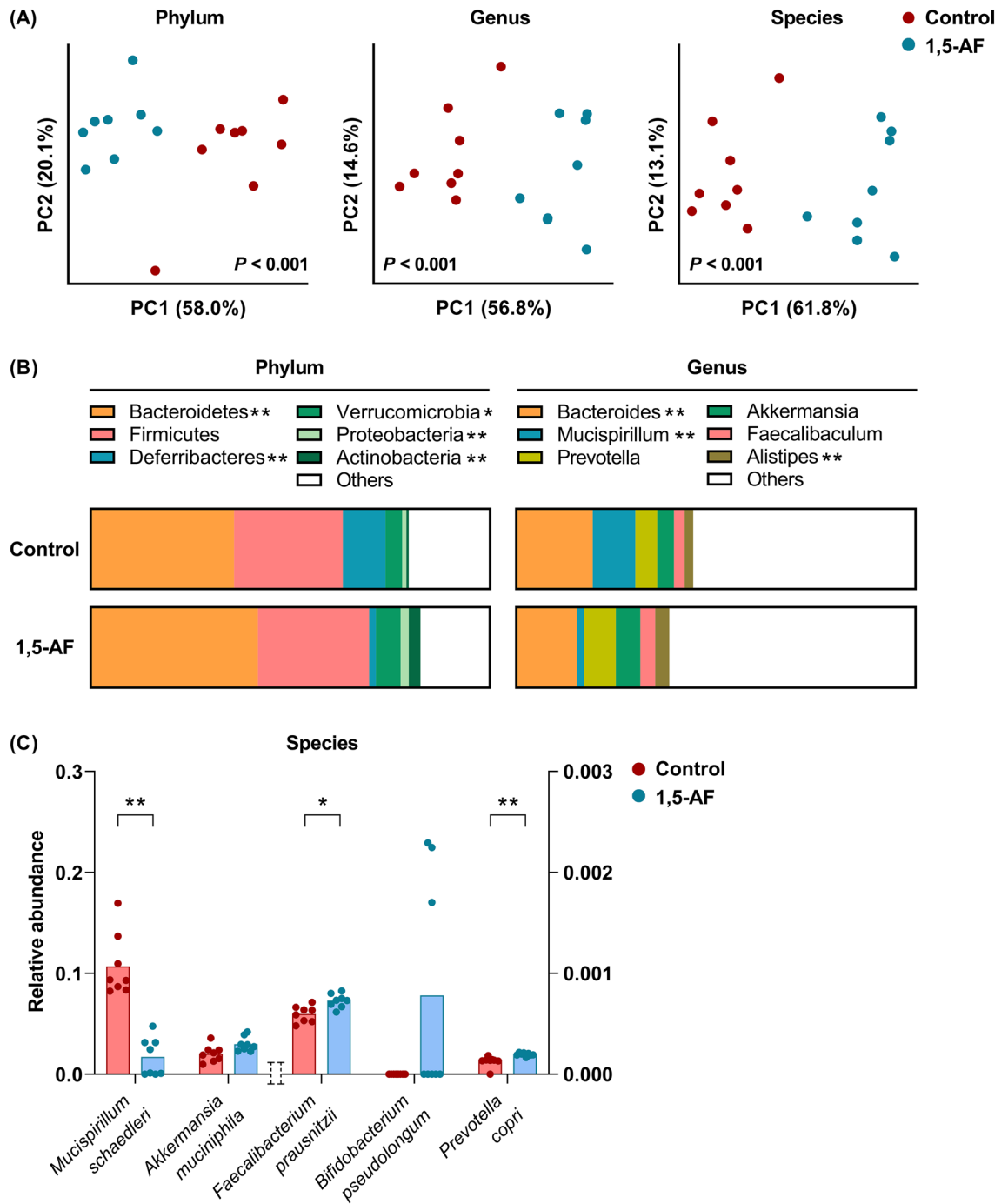


Figure 1. Dietary supplementation with 1,5-AF modulated gut microbiota in mice. **(A)** Principal coordinate analysis (PCoA) indicated that mice supplemented with or without 1,5-AF (n = 8, each group) were distinguishable by the structure of the gut microbiota at the phylum, genus, and species levels. Statistical significance of the differences between groups was calculated by PERMANOVA. **(B)** At the phylum level, relative abundance of Bacteroidetes was high in mice supplemented with 1,5-AF. Within Bacteroidetes, the genus *Bacteroides* was less abundant, whereas genera *Prevotella* and *Alistipes* were more abundant in mice supplemented with 1,5-AF. Mean values of the mice in each group (n = 8) are shown. **(C)** Relative abundances of each microbial species in mice supplemented with 1,5-AF (n = 8) were compared with those in control mice (n = 8). *M. schaedleri* and *A. muciniphila* are assigned to the left axis and *F. prausnitzii*, *B. pseudolongum*, and *P. copri* are assigned to the right axis. Bars indicate mean values. * $P < 0.05$ and ** $P < 0.01$, determined by Mann–Whitney test with FDR adjustment for multiple comparison.

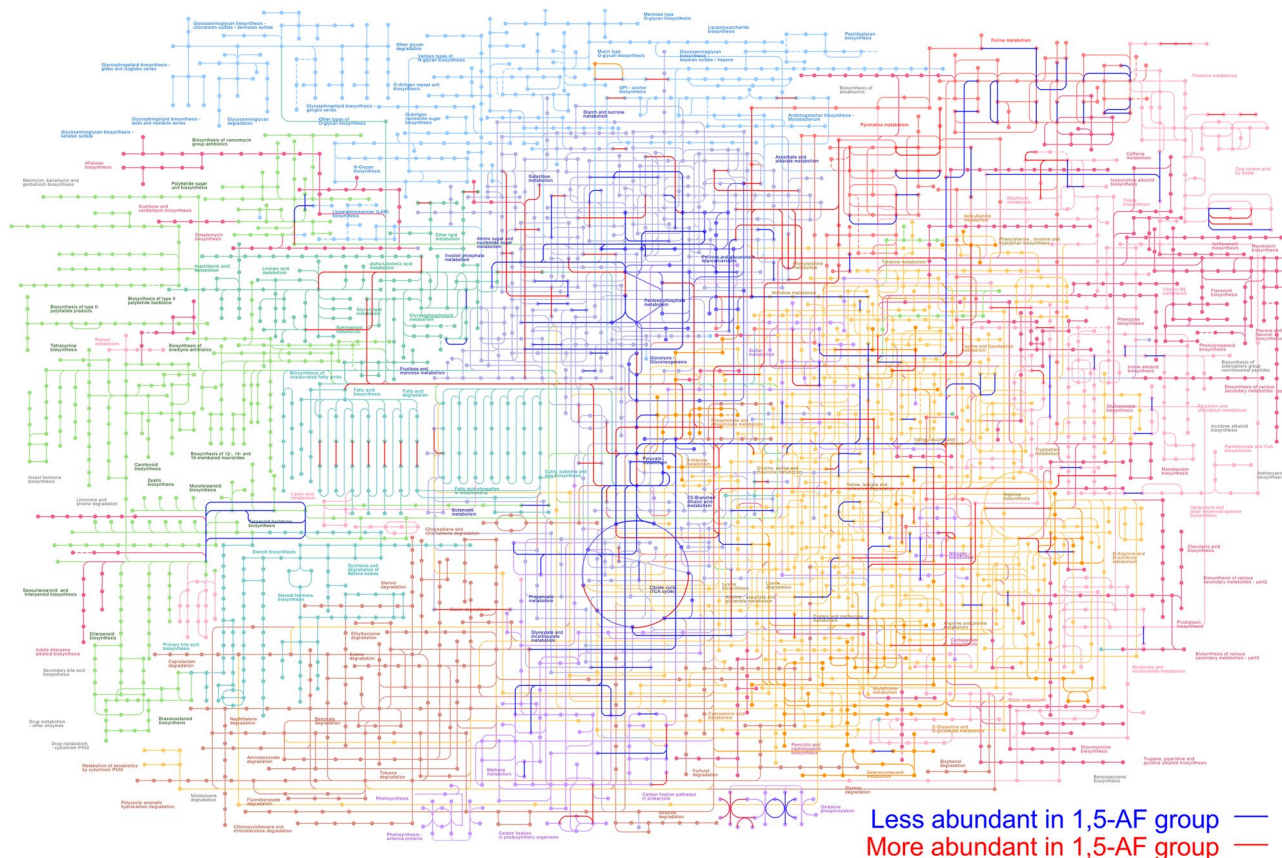


Figure 2. Comprehensive map of the metabolic pathways upregulated or downregulated by dietary supplementation with 1,5-AF. The metagenomic reads of the gut microbiota in mice treated with or without 1,5-AF ($n=8$ in each group) were mapped against a functional reference database, and gene abundances were determined as reads per kilobase of exon per million mapped sequence reads (RPKM). The differences in RPKM values between the 1,5-AF-treated mice and the control mice were analysed using White's non-parametric t -test with FDR adjustment for multiple comparison. Significantly more or less abundant genes in the gut microbiota of the 1,5-AF-treated mice are shown with red or blue lines, respectively. The metabolic map was created using the Kyoto Encyclopedia of Genes and Genomes (KEGG) mapper²⁸ with the copyright permission.

tion of butyrate-producing bacteria *Faecalibacterium prausnitzii* was higher in mice supplemented with 1,5-AF (Fig. 1C), although that of *Roseburia* or *Butyrivibrio* was not. The proportion of *Bifidobacterium* was also high in some mice supplemented with 1,5-AF.

Genes associated with nicotinamide adenine dinucleotide (NAD) biosynthesis were enriched in the gut microbiota of mice supplemented with 1,5-AF. To profile the functional consequences of the gut microbial modification induced by 1,5-AF, the metagenomic sequencing data were mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology database. In this analysis, $24,940,051 \pm 3,866,382$ reads were mapped to 3812 bacterial genes (Supplementary Material 2). Among these, 249 genes were significantly less abundant, and 232 genes were significantly more abundant in mice supplemented with 1,5-AF (Fig. 2). The more abundant genes included those associated with NAD biosynthesis (Fig. 3), such as *nadB* ($P < 0.05$) and *nadA* ($P < 0.05$).

Discussion

In this study, we analysed the effects of 1,5-AF supplementation on the gut microbiota in healthy young mice. Supplementation with 1,5-AF altered (i) the composition of the gut microbiota, enriching *F. prausnitzii* and *Bifidobacterium*; and (ii) the metabolomic profile of the gut microbiota, enriching the genes associated NAD biosynthesis.

Nicotinamide and NAD are vitamins that participate in numerous redox reactions, including glycolysis, pyruvate-to-lactate and pyruvate-to-acetyl-CoA interconversions, β -oxidation, and oxidative phosphorylation. NAD also plays crucial roles in the regulation of metabolism and mitochondrial functions by acting as a co-substrate for NAD-dependent deacetylases (sirtuins)²². NAD can be produced either through de novo synthesis or through salvage pathways from precursor molecules, including nicotinamide. In the de novo synthesis pathway, NAD is synthesized in sequential steps, starting from aspartate or tryptophan²³. In the present study, the genes encoding

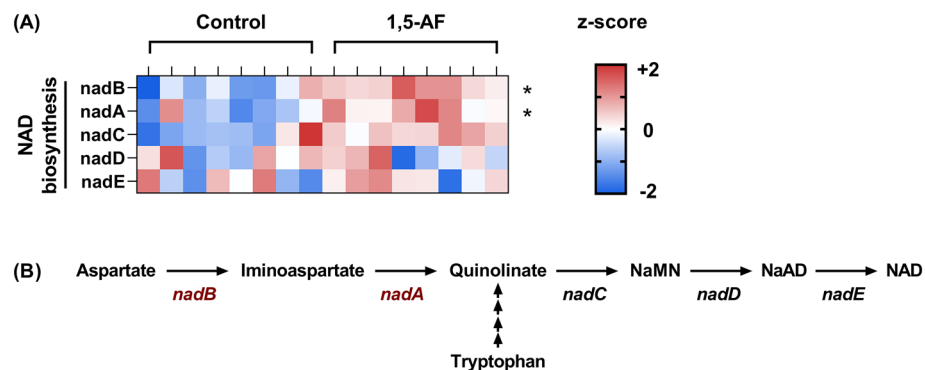


Figure 3. Genes associated with NAD biosynthesis were enriched in the gut microbiota of mice supplemented with 1,5-AF. (A) Relative gene abundances (z-scores) in NAD biosynthesis are shown; red and blue indicate high and low abundances, respectively. *nad*: nicotinamide adenine dinucleotide. $*P < 0.05$, determined by White's non-parametric *t*-test with FDR adjustment for multiple comparison. (B) Schematic diagram of biosynthesis of NAD is shown. The genes encoding enzymes involved in the first two steps of the de novo synthesis of NAD from aspartate, were significantly enriched in the mice supplemented with 1,5-AF. NaMN: nicotinic acid mononucleotide. NaAD: nicotinic acid dinucleotide.

nadB (L-aspartate oxidase) and *nadA* (quinolinate synthase), proteins involved in the first two enzymatic steps of the de novo synthesis of NAD from aspartate, were significantly increased in the mice supplemented with 1,5-AF (Fig. 3B). Because NAD can be synthesized from aspartate even in the absence of oxygen²³, this pathway might be important in anaerobic NAD biosynthesis in the gut microbiota.

F. prausnitzii is a metabolically active commensal bacterium in healthy adults, and is considered a potential probiotic bacterium for human gastrointestinal diseases²⁴. It is one of the most abundant butyrate-producing bacteria in the gastrointestinal tract, and by producing butyrate, *F. prausnitzii* may affect the intestinal-cell life cycle, pathogen resistance, and cancer progression. *Bifidobacterium* is considered a butyrogenic bacterium, and preferentially coexists with butyrate-producing bacteria such as *F. prausnitzii*²⁵. During carbohydrate fermentation, *Bifidobacterium* produces acetate and lactate, which can be converted, in turn, to butyrate by *F. prausnitzii* in cross-feeding interactions. In this study, the relative abundance of both *F. prausnitzii* and *Bifidobacterium* was higher in mice fed with 1,5-AF diet compare with those fed with control diet, suggesting that 1,5-AF can be used to modulate the gut microbiota to counteract gastrointestinal diseases.

Our study had several limitations. First, it is unclear whether 1,5-AF directly stimulates the growth of specific bacteria associated with wellbeing or indirectly affects them through various host responses in the gastrointestinal tract²⁶. Radioisotope tracing experiments showed that 63% of orally administered 1,5-AF was absorbed and renally excreted, 11% was found in the expired air, and 5% was found in the faeces²⁷, suggesting that 1,5-AF may influence gut microbiota both directly and indirectly. Second, it is unclear whether the relative difference between the 1,5-AF group and the control (erythritol) group was due to the stimulatory effect of 1,5-AF or the inhibitory effect of erythritol. Third, dose–response effects of 1,5-AF were not examined in this study, and thus, the optimum dosage is uncertain. Fourth, it is unclear whether the effects of 1,5-AF are actually beneficial, neutral, or potentially harmful, because we used healthy young mice, which showed little change in their condition during the study period. In this context, we are planning to examine the effects of 1,5-AF on the gut microbiota of mice with age-associated diseases and inflammatory diseases. Further studies are required to clarify the potential effects of 1,5-AF in healthy and diseased organisms.

Conclusions

Dietary supplementation with 1,5-AF modulated gut microbiota in mice. Genes associated with NAD biosynthesis were enriched in the gut microbiota of mice supplemented with 1,5-AF.

Data availability

All data generated or analysed during this study are included in this published article and its Supplementary Information files.

Received: 25 March 2021; Accepted: 17 September 2021

Published online: 04 October 2021

References

- Shreiner, A. B., Kao, J. Y. & Young, V. B. The gut microbiome in health and in disease. *Curr. Opin. Gastroenterol.* **31**(1), 69–75 (2015).
- Lynch, S. V. & Pedersen, O. The human intestinal microbiome in health and disease. *N. Engl. J. Med.* **375**(24), 2369–2379 (2016).
- Bar, N. *et al.* A reference map of potential determinants for the human serum metabolome. *Nature* **588**(7836), 135–140 (2020).
- Chatelier, E. *et al.* Richness of human gut microbiome correlates with metabolic markers. *Nature* **500**(7464), 541–546 (2013).
- Qin, J. *et al.* A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature* **490**(7418), 55–60 (2012).

6. Suzuki, T. A. & Ley, R. E. The role of the microbiota in human genetic adaptation. *Science* **370**(6521), 1 (2020).
7. Yatsunenko, T. *et al.* Human gut microbiome viewed across age and geography. *Nature* **486**(7402), 222–227 (2012).
8. Wu, G. D. *et al.* Linking long-term dietary patterns with gut microbial enterotypes. *Science* **334**(6052), 105–108 (2011).
9. Kovatcheva-Datchary, P. *et al.* Dietary fiber-induced improvement in glucose metabolism is associated with increased abundance of *Prevotella*. *Cell Metab.* **22**(6), 971–982 (2015).
10. De Vadder, F. *et al.* Microbiota-produced succinate improves glucose homeostasis via intestinal gluconeogenesis. *Cell Metab.* **24**(1), 151–157 (2016).
11. Pedersen, H. K. *et al.* Human gut microbes impact host serum metabolome and insulin sensitivity. *Nature* **535**(7612), 376–381 (2016).
12. De Filippis, F. *et al.* Distinct genetic and functional traits of human intestinal *Prevotella* copri strains are associated with different habitual diets. *Cell Host Microbe* **25**(3), 444–453.e443 (2019).
13. Schott, E. M. *et al.* Targeting the gut microbiome to treat the osteoarthritis of obesity. *JCI Insight* **3**(8), 1 (2018).
14. Blacher, E. *et al.* Potential roles of gut microbiome and metabolites in modulating ALS in mice. *Nature* **572**(7770), 474–480 (2019).
15. Fiskesund, R. *et al.* 1,5-anhydro-D-fructose and its derivatives: biosynthesis, preparation and potential medical applications. *Planta Med.* **76**(15), 1635–1641 (2010).
16. Yamaji, K., Sarker, K. P., Maruyama, I. & Hizukuri, S. Antioxidant effects of 1,5-anhydro-D-fructose, a new natural sugar, in vitro. *Planta Med.* **68**(1), 16–19 (2002).
17. Meng, X. *et al.* 1,5-Anhydro-D-fructose attenuates lipopolysaccharide-induced cytokine release via suppression of NF-kappaB p65 phosphorylation. *Biochem. Biophys. Res. Commun.* **380**(2), 343–348 (2009).
18. Meng, X. *et al.* 1,5-Anhydro-D-fructose: A natural antibiotic that inhibits the growth of gram-positive bacteria and microbial biofilm formation to prevent nosocomial infection. *Exp. Ther. Med.* **2**(4), 625–628 (2011).
19. Mei, J., Yu, S. & Ahrén, B. A 90-day toxicological evaluation of 1,5-anhydro-d-fructose in Sprague-Dawley rats. *Drug Chem Toxicol* **28**(3), 263–272 (2005).
20. White, J. R., Nagarajan, N. & Pop, M. Statistical methods for detecting differentially abundant features in clinical metagenomic samples. *PLoS Comput Biol* **5**(4), e1000352 (2009).
21. Storey, J. D. & Tibshirani, R. Statistical significance for genomewide studies. *Proc. Natl. Acad. Sci. USA* **100**(16), 9440–9445 (2003).
22. Katsyuba, E. & Auwerx, J. Modulating NAD⁺ metabolism, from bench to bedside. *EMBO J.* **36**(18), 2670–2683 (2017).
23. Gazzaniga, F., Stebbins, R., Chang, S. Z., McPeck, M. A. & Brenner, C. Microbial NAD metabolism: Lessons from comparative genomics. *Microbiol. Mol. Biol. Rev.* **73**(3), 529–541 (2009).
24. Miquel, S. *et al.* Faecalibacterium prausnitzii and human intestinal health. *Curr. Opin. Microbiol.* **16**(3), 255–261 (2013).
25. Rivière, A., Selak, M., Lantin, D., Leroy, F. & De Vuyst, L. Bifidobacteria and butyrate-producing colon bacteria: Importance and strategies for their stimulation in the human gut. *Front. Microbiol.* **7**(979), 1 (2016).
26. Ahrén, B., Holst, J. J. & Yu, S. 1,5-Anhydro-d-fructose increases glucose tolerance by increasing glucagon-like peptide-1 and insulin in mice. *Eur. J. Pharmacol.* **397**(1), 219–225 (2000).
27. Yu, S., Mei, J. & Ahrén, B. Basic toxicology and metabolism studies of 1,5-anhydro-D-fructose using bacteria, cultured mammalian cells, and rodents. *Food Chem. Toxicol.* **42**(10), 1677–1686 (2004).
28. Kanehisa, M. & Goto, S. KEGG: kyoto encyclopedia of genes and genomes. *Nucl. Acids Res.* **28**(1), 27–30 (2000).

Acknowledgements

We thank all the staff members at the Institute of Laboratory Animal Sciences, Research Support Center, Kagoshima University, for maintaining the animals in good condition. We also thank Janine Miller, PhD, from Edanz Group (<https://en-author-services.edanz.com/ac>) for editing a draft of this manuscript.

Author contributions

T.I. designed the experimental protocol and wrote the manuscript. T.T., S.T., and S.O. participated in the animal experiments. I.M. critically appraised the manuscript. All authors read and approved the final manuscript.

Funding

This work was supported, in part, by a research grant from the Japan Society for the Promotion of Science (Grants-in-Aid 21H03033).

Competing interests

TI, ST, SO, and IM hold endowed faculty positions at Kagoshima University and receive research funding from Yakuryo Kaihatsu Co., Ltd, a vendor of dietary supplements, including 1,5-AF. TT declares that he has no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-021-99052-y>.

Correspondence and requests for materials should be addressed to T.I.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2021