HOKKAIDO UNIVERSITY

Title	Murine fecal microRNAs alter the composition of cultured gut microbiota
Author(s)	Ohsaka, Fumina; Yamaguchi, Mayuko; Teshigahara, Yuka et al.
Citation	Biochemical and biophysical research communications, 685, 149184 https://doi.org/10.1016/j.bbrc.2023.149184
Issue Date	2023-10-29
Doc URL	https://hdl.handle.net/2115/93256
Rights	©2023. This manuscript version is made available under the CC-BY-NC-ND 4.0 license http://creativecommons.org/licenses/by-nc-nd/4.0/
Rights(URL)	https://creativecommons.org/licenses/by-nc-nd/4.0/
Type	journal article
File Information	Ohsaka-Yamaguchi2023BBRC-e.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Murine fecal microRNAs alter the composition of cultured gut microbiota

Fumina Ohsaka [a,ł], Mayuko Yamaguchi [b,ł], Yuka Teshigahara [b], Moeka Yasui [b], Eisuke Kato [a] and Kei Sonoyama [a,*]

[a] Research Faculty of Agriculture, Hokkaido University, Sapporo 060-8589 Japan

[b] Graduate school of Agriculture, Hokkaido University, Sapporo 060-8589 Japan

[ł] These authors contributed equally to this work.

*Corresponding author: Kei Sonoyama

Laboratory of Food Biochemistry,

Research Faculty of Agriculture, Hokkaido University

Kita-9 Nishi-9, Kita-ku, Sapporo 060-8589, Japan

Tel & Fax: +81-11-706-2496; E-mail: ksnym@agr.hokudai.ac.jp

Abstract

Fecal microRNAs (miRNAs) derived from intestinal epithelial cells have been suggested to influence gut microbiota homeostasis. The present study examined whether fecal miRNAs alter the structure of cultured gut microbiota. Fecal bacteria isolated from murine cecal contents were cultured for 24 h under anaerobic conditions. Supplementation with fecal small RNAs isolated from cecal contents altered the structure of cultured fecal microbiota as assessed by 16S rRNA gene sequence analysis. In particular, fecal small RNAs increased *Enterococcus* spp. Fractionation of fecal small RNAs by ultrafiltration showed that small RNAs smaller than 10 kDa significantly increased enterococci compared to those larger than 10 kDa, as assessed by quantitative PCR, suggesting that the increase in enterococci by fecal small RNAs can mainly be attributed to miRNAs. Negative control miRNA that has low homology to miRNA sequences of human, mouse, and rat, failed to increase enterococci. Therefore, the findings from the present study employing cultured fecal bacteria suggest that fecal small RNAs, most likely host-derived miRNAs, alter gut microbiota structure by expanding enterococci in a sequence-dependent manner.

Keywords: Gut microbiota, MicroRNA, *Enterococcus*

1. Introduction

MicroRNAs (miRNAs) are small (~22 nucleotides (nt) in length) single-stranded non-coding RNA species [1, 2]. They bind to the 3′ untranslated region (UTR) of their target mRNA, resulting in silencing of gene expression through mRNA degradation or translational repression [3]. This miRNA-mediated gene silencing regulates diverse physiological processes. In addition, many studies have reported that miRNAs play a role in mediating the influence of gut microbiota on numerous aspects of host physiology, including regulation of the intestinal immune system [4, 5].

Intriguingly, host-derived miRNAs have been shown to influence homeostasis of the gut microbiota. By employing mice lacking Dicer, an RNase III enzyme essential for mature miRNA synthesis, Liu *et al.* [6] demonstrated that miRNAs in the intestinal lumen are derived from intestinal epithelial cells and miRNA deficiency in the lumen alters the structure of the gut microbiota. The host maintains gut microbiota homeostasis through secretion of immunoglobulin A antibodies and antimicrobial peptides into the intestinal lumen [7, 8]. Thus, host miRNAs could be an important additional factor for maintaining gut microbiota homeostasis.

The present study investigated whether host-derived miRNAs alter the structure of the gut microbiota homeostasis using cultured gut microbiota.

2. Materials and methods

2.1. Animal care

All study protocols were pre-approved by the Animal Use Committee of Hokkaido University (approval no. 19-0017), and all mice were maintained in accordance with the guidelines for the care and use of laboratory animals of Hokkaido University. Female C57BL/6J JmsSlc mice (six weeks of age) were purchased from Japan SLC (Hamamatsu, Japan). Mice were housed in standard plastic cages in a temperature-controlled (23 ± 2°C) room under a 12-h light/dark cycle. They were allowed free access to the AIN-93G pellet diet (D10012G, Research Diets, New Brunswick, NJ) and tap water.

2.2. Isolation and culture of fecal bacteria

Mice were euthanized by cervical dislocation under sevoflurane anesthesia. Following a laparotomy, the cecal contents were collected and suspended in 20-fold 0.05% (w/v) L-cysteine in buffered peptone water (Oxoid, Basingstoke, UK) and vortexed until homogeneous. Next, 100 µL of the suspension was inoculated into 900 uL of mucin medium contained within each well of a 24-well plate. The mucin medium was composed of 3.5 g/L K_2HPO_4, 10.9 g/L KH_2PO_4, 2 g/L $NaHCO_3$, 2 g/L yeast extract (Becton Dickinson, Sparks, MD, USA), 2 g/L polypeptone (Nihon Pharmaceutical, Tokyo, Japan), 1 g/L mucin (from porcine stomach, Fuji Film Wako Pure Chemical, Osaka, Japan), 0.5 g/L L-cysteine, and 2 mL/L Tween80 [9]. The cultures were then supplemented with either fecal small RNAs isolated as described below, negative control miRNA (AccuTarget™ miRNA negative control, mimic #1, Bioneer, Daejeon, South Korea) or vehicle (RNase-free water), and incubated with shaking at 120 rpm under anaerobic conditions using the AnaeroPack system (Mitsubishi Gas Chemical, Tokyo, Japan) at 37°C for 24 h (**Fig. 1A**). The negative control miRNA has low homology with miRNA sequences from human, mouse, and rat. The culture medium and 0.05% (w/v) L-cysteine in buffered peptone water were pre-treated with the AnaeroPack system for 24 h before use. Bacterial growth was assessed by measuring the optical density of the cultures at 600 nm (OD600) using a spectrophotometer (GeneQuant pro, Pharmacia Biotech, Cambridge, UK). After centrifuging the cultures at 15,000 rpm for 10 min, pH and organic acids were measured in the supernatant [10], and DNA was isolated from the pellet, as described below.

2.3. Isolation of small RNAs, including miRNAs, from cecal contents

A separate group of mice were euthanized by cervical dislocation under sevoflurane anesthesia. Following a laparotomy, the cecal contents were collected. Small RNAs, including miRNAs, were isolated from cecal contents using a MicroRNA Isolation Kit (BioChain, Hayward, CA, USA) according to the manufacturer's instructions. The concentration of small RNAs in each sample was determined by the Qubit microRNA Assay Kit (Thermo Fisher Scientific, San Jose, CA, USA) using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific)

according to the manufacturer's instructions. The small RNA samples were stored at -80°C until further use.

2.4. DNA isolation from cultured bacteria and quantitative PCR (qPCR) analysis

Following the manufacturer's instructions, DNA was isolated from the bacterial pellet using a QIAamp Fast DNA Stool Mini kit (Qiagen, Tokyo, Japan). DNA samples served as the template for qPCR of the 16S rRNA gene fragments to estimate the number of total bacteria and *Enterococcus* spp. We conducted qPCR using the GeneAce SYBR qPCR Mix α No ROX (Nippon Gene, Toyama, Japan) with a Thermal Cycler Dice TP800 (Takara Bio, Otsu, Japan). The reaction conditions were 95°C for 10 min, followed by 45 cycles at 95°C for 30 s and 60°C for 60 s. A melting curve analysis was performed after amplification to assess the specificity of qPCR. Primer sequences for qPCR are shown in **Supplementary Table 1**. For qPCR standards, a subcloned 16S rRNA gene fragment was prepared from *Blautia coccoides* (ATCC 29236) and *Enterococcus faecalis* AHU2248 for total bacteria and genus *Enterococcus* respectively, as previously described [11]. *E. faecalis* AHU2248 was provided by the Laboratory of Applied Molecular Microbiology in the Research Faculty of Agriculture of Hokkaido University.

2.5. Analysis of gut microbiota using 16S rRNA gene sequencing data

The V3-V4 regions of the 16S rRNA gene were amplified by PCR using a universal primer set (341F and 805R) with Illumina overhang adaptor sequences. The PCR reaction was conducted in a total volume of 50 μL containing 2 ng/mL DNA, 0.3 pmol/mL of each primer, 0.4 mM dNTPs, 0.01 U/mL KOD Fx Neo DNA polymerase (Toyobo, Osaka, Japan), and 1×KOD Fx Neo PCR buffer. PCR conditions were as follows: initial denaturation at 94°C for 2 min, followed by 25 cycles at 98°C for 10 s, 55°C for 30 s, with final extension at 68°C for 7 min. The amplicons were purified with AMPure XP Beads (Beckman coulter, Indianapolis, IN, USA). A second PCR reaction was conducted to attach barcode indices and adapters using the Herculase II Fusion DNA Polymerase Nextera XT Index Kit V2 (Illumina, San Diego, CA, USA), and 300 bp paired-end sequencing was performed on an Illumina MiSeq platform at Genome-Lead Co. (Takamatsu, Japan). Sequence data analysis was performed

with the QIIME2 platform (version 2022.2) [12]. After demultiplexing and primer cutting, the sequences were qualified through filtering, denoising, merging and chimera removal using the DADA2 pipeline [13]. Taxonomic classification was assigned to amplicon sequence variants using the SILVA release 132 with taxonomic classification at a confidence level >99% [14]. Chao1 and Shannon α-diversity indexes were calculated with QIIME2 scripts. β-diversity was estimated using the unweighted and weighted UniFrac metrics to calculate the distances between the samples. The results were visualized by principal coordinate analysis (PCoA) plots and statistically analyzed using permutational analysis of variance (PERMANOVA). Analysis of α- and β-diversities was performed by rarefying the feature table at a consistent sample depth of 10,000. A linear discriminant analysis effect size (LEfSe) analysis [15] and analysis of communities in the microbiota (ANCOM) [16] were performed to identify taxa that were different between groups.

2.6. Uptake of Cy3-labeled small RNAs by cultured fecal bacteria

Small RNAs isolated from cecal contents were labeled with Cy3 using a ULS microRNA Labeling Kit (Kreatech Diagnostics, Amsterdam, the Netherlands), following the manufacturer's instructions. Fecal bacteria were cultured as described above and supplemented with Cy3-labeled small RNAs (final concentration, 1 μM as 20-nt miRNAs) for 4 h. Bacteria were then pelleted and subjected to Gram staining. Alternatively, bacteria were stained with DAPI using a Bacstain DAPI solution (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions and then observed under a fluorescent microscope (BX40, Olympus, Tokyo, Japan).

2.7. Ultrafiltration and analysis of fecal small RNAs

Fecal small RNAs were separated by centrifugal ultrafiltration using Vivaspin 500 (10,000 molecular weight cut-off, Sartorius, Göttingen, Germany). For the concentrates and filtrates, the size distribution of small RNAs was assessed using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA), performed by Toray Industries (Tokyo, Japan). The concentrates and filtrates were also subjected to fecal bacterial culture experiments as described above.

2.8. Statistical analysis

Results are presented as standard box plots with median and interquartile ranges and minimum and maximum values. To compare the mean values of the two groups, a Welch's *t*-test was used. Dunnett's T3 multiple comparison test following Brown-Forsythe one-way ANOVA was used to compare the mean values of the three groups. Data were analyzed using GraphPad Prism software for Macintosh (version 10, GraphPad Software, San Diego, CA). *P* values of <0.05 were considered statistically significant.

3. Results

3.1. Comparison of microbiota structure between fecal bacteria isolated from cecal contents and cultured fecal bacteria

Analysis of the 16S rRNA gene sequence showed that the Chao1 index was significantly lower, and the Shannon index tended to be lower in cultured microbiota compared to the isolated microbiota (**Supplementary Figs. 1A** and **1B**, respectively). The β-diversity of microbiota was determined based on the PCoA using different statistical metrics including the unweighted UniFrac distance, a phylogenetic metric that considers the presence or absence of taxa (**Supplementary Fig. 1C**), and the weighted UniFrac distance, a phylogenetic metric that accounts for abundances of observed taxa (**Supplementary Fig. 1D**). Significant differences were observed in both unweighted and weighted UniFrac distances between the groups, as determined by PERMANOVA analysis. At the phylum level, Bacillota, Bacteroidota, Desulfobacterota, and Deferribacterota were predominant in isolated fecal microbiota, whereas Pseudomonadota, Bacteroidota, Bacillota, Verrucomicrobiota, and Desulfobacterota were predominant in cultured microbiota (**Supplementary Fig. 1E**).

3.2. Fecal small RNAs promote the growth and fermentation of cultured fecal bacteria

To test whether fecal small RNAs influence the gut microbiota, small RNAs isolated from murine cecal contents were added to the culture of fecal bacteria at a final concentration of 0.5 μM of 20-nt miRNAs (**Fig. 1A**). OD600 values of the culture supplemented with vehicle were significantly higher at 24 h compared to the values at the start of culture (**Fig. 1B**).

Furthermore, at 24 h of culture, OD600 values were significantly higher in the culture supplemented with fecal small RNAs compared to the culture supplemented with vehicle. The pH of the culture supernatant was significantly lower at 24 h of culture compared to initial values, both in the culture supplemented with vehicle and that supplemented with fecal small RNAs (**Fig. 1C**). The concentrations of short-chain fatty acids (SCFAs) namely acetate, propionate, and butyrate, which are the principal bacterial fermentation products, were significantly higher in the supernatants from the cultures supplemented with vehicle and fecal small RNAs at 24 h of culture compared to the beginning of culture (0 h). (**Fig. 1D**). In addition, SCFA concentrations at 24 h were significantly higher in the culture supplemented with fecal small RNAs compared to culture supplemented with vehicle.

3.3. Fecal small RNAs are incorporated into cultured fecal bacteria

Cy3-labeled small RNAs isolated from cecal contents were added to the culture for 4 h at a final concentration of 1 μM of 20-nt miRNAs to test whether fecal small RNAs were incorporated into cultured fecal bacteria. The Cy3 signal was observed in several but not all bacterial cells (**Fig. 1E**).

3.4. Fecal small RNAs alter the structure of cultured fecal microbiota

Bacteria isolated from cecal contents were cultured for 24 h in medium supplemented with small RNAs isolated from cecal contents containing a final concentration of 0.5 μM of 20-nt miRNAs. According to the Chao1 index, there were no significant differences in species richness between the groups (**Fig. 2A**). In contrast, the Shannon index showed significantly higher species evenness in the culture supplemented with fecal small RNA compared to the culture supplemented with vehicle (**Fig. 2B**). Regarding β-diversity, there was a significant difference in weighted UniFrac distance between the groups as determined by PERMANOVA analysis (**Figs. 2C** and **2D**). No differences were observed in microbiota structure at the phylum level between the groups (**Supplementary Fig. 2**). At the genus level, LEfSe analysis showed that 9 bacterial taxa were enriched in the culture supplemented with fecal small RNAs and 7 bacterial taxa were enriched in the culture supplemented with vehicle (**Fig. 2E**). In addition, ANCOM analysis revealed significantly higher relative abundance of enterococci in

the culture supplemented with fecal small RNAs compared to the culture supplemented with vehicle (**Fig. 2F**). Since the two methods yielded different results, we proceeded with investigation of enterococci that were cross-validated by both LEfSe and ANCOM methods. qPCR analysis showed that while the number of total bacteria did not differ between the groups (**Fig. 2G**), the number and ratio of enterococci tended to be higher in the culture supplemented with fecal small RNAs compared to the culture supplemented with vehicle (**Figs. 2H** and **2I**).

3.5. miRNAs are responsible for the effects of fecal small RNAs on cultured fecal bacteria

Bioanalyzer analysis of small RNAs isolated from cecal contents showed a major peak of size ~200 nt in the unfractionated small RNAs (**Figs. 3A**). The size distribution of small RNAs in the concentrate following ultrafiltration using Vivaspin (10,000 molecular weight cut-off) was similar to that of the unfractionated small RNAs (**Fig. 3B**). In contrast, in the filtrate, this peak was absent and smaller RNAs were dominant (**Fig. 3C**). The unfractionated small RNAs, concentrate, and filtrate were added to the culture of fecal bacteria at the same RNA concentration (0.8 μM of 20-nt miRNAs), and the abundance of enterococci was assessed by qPCR. The increase in the ratio of enterococci was significantly higher in the culture supplemented with the filtrate compared to either the culture supplemented with the unfractionated small RNAs or that supplemented with concentrate. (**Fig. 3D**).

3.6. Effects of fecal miRNAs on cultured fecal bacteria depend on their sequences

To further investigate the specificity of the actions of fecal small RNAs on cultured fecal bacteria, we compared the actions of fecal small RNAs and the negative control miRNA, which has low homology with the miRNA sequence of human, mouse, and rat. Fecal bacteria were supplemented with either small RNAs isolated from cecal contents, negative control miRNA, or vehicle for 24 h. qPCR analysis showed that the number of total bacteria did not differ between the groups (**Fig. 4A**). In contrast, the number and the ratio of enterococci were significantly higher in the culture supplemented with fecal small RNAs compared to the cultures supplemented with negative control miRNA or vehicle (**Figs. 4B** and **4C**). The pH value in the culture supernatant was significantly lower in the culture supplemented with fecal

small RNAs compared to the cultures supplemented with negative control miRNA or vehicle (**Fig. 4D**). Although the concentration of acetate and propionate in the culture supernatant tended to be higher in the culture supplemented with fecal small RNAs compared to the cultures supplemented with negative control miRNA or vehicle (**Fig. 4E**), the concentration of butyrate did not differ between the groups. Furthermore, there were no differences in the number and ratio of enterococci, pH values, and SCFA concentrations between the culture supplemented with negative control miRNA and the culture supplemented with the vehicle.

4. Discussion

To culture murine fecal bacteria, the present study employed a medium in which mucin is the sole carbon source, to mimic conditions in the colon [9]. An increase in OD600 values after 24 h of culture, indicated bacterial growth, and a decrease in pH values and increase in the concentrations of SCFAs in the culture supernatant indicated the fermentation of cultured bacteria. These results suggest that fecal bacteria were successfully cultured in this medium. Although the structure of the cultured fecal microbiota does not fully reflect the structure of the gut microbiota *in vivo*, the culture system in the present study could be a useful *in vitro* model to investigate the influence of host-derived miRNAs on the gut microbiota.

The present study demonstrated that supplementation of cultured fecal microbiota with fecal small RNAs altered their structure, particularly by increasing the ratio of the genus *Enterococcus*. In addition, by fractionating fecal small RNAs by ultrafiltration, we found that small RNAs smaller than 10 kDa significantly increased *Enterococcus* spp. compared to RNA species larger than 10 kDa. Since miRNAs are ~22-nt single-stranded RNA species, it is likely that the increase in enterococci due to fecal small RNAs is primarily mediated by miRNAs. However, we cannot disregard the possibility that tRNA-derived small RNAs could contribute to these effects since tRNA-derived fragments are approximately 14-30 nt in length, similar to miRNAs, and may have identical functions to miRNAs in inhibiting mRNA translation [17]. We could not distinguish between the effects of tRNA-derived fragments and miRNAs on the cultured fecal microbiota in this study.

To test whether the increase in the ratio of enterococci in the cultured fecal microbiota is related to the sequences of fecal small RNAs, we compared the effects of fecal small RNAs with those of the negative control miRNA, which has low homology with miRNA sequences of human, mouse, and rat. According to the manufacturer, the negative control has been analyzed by BLAST against all human, mouse, and rat genomic sequences and against miRNA sequences in the current miRBase database. The present study clearly showed that supplementation with the negative control miRNA failed to increase the ratio of enterococci in the cultured fecal microbiota, suggesting a sequence-dependent action of fecal small RNAs on enterococci. Liu *et al.* [6] demonstrated that fecal miRNAs enter *Escherichia coli* and *Fusobacterium nucleatum* and regulate bacterial gene transcripts in a sequence-specific manner thereby affecting growth. In addition, Liu *et al.* [18] showed regulation of gut microbes by specific miRNA. They found that miR-30d-5p regulates the expression of the β-galactosidase gene in *A. muciniphila*, which expands this microbe in the gut. Their findings suggested that specific miRNAs derived from the host influence the growth of distinct bacteria by regulating the expression of particular genes. Although the present study showed that Cy3-labeled fecal small RNAs were incorporated into cultured fecal bacteria, it remains to be determined whether fecal small RNAs promote enterococci growth by specifically regulating gene expression. In addition, it is unclear whether the observed alteration of microbiota structure is associated with the uptake of small RNAs by specific bacterial taxa.

We observed that the concentration of SCFAs in the culture supernatant of fecal bacteria was influenced by supplementation with fecal small RNAs, but not by supplementation with negative control miRNA. Specific bacterial taxa are known to produce SCFAs in the gut. For example, acetate producers including *Prevotella* spp., *Ruminococcus* spp., *Bifidobacterium* spp., *Bacteroides* spp., *Clostridium* spp., *Streptococcus* spp., and *A. muciniphila,* are known to be widely distributed among the gut microbiota [19]. However, these bacterial taxa were not included in the different abundant taxa between the groups, detected by LEfSe and ANCOM analyses in the present study. This suggests the possibility that fecal small RNAs alter the activity of acetate fermentation within each acetate producer rather than the

abundance of these bacteria. Further studies are needed to test whether specific small RNAs target the genes responsible for SCFA production.

Enterococcus spp. colonize the digestive system as commensals and modulate the immune system of the host [20]. However, enterococci are also seen as opportunistic pathogens. The physiological significance of host-derived miRNAs that increase enterococci in the gut microbiota requires further studies and discussion.

In conclusion, the present data employing cultured fecal bacteria suggest that fecal small RNAs, that are most likely host-derived miRNAs, alter gut microbiota structure by promoting the growth of enterococci. Growing evidence suggests that miRNA-induced gene silencing plays a significant role in the regulation of host physiology by gut microbiota. As shown in the present study, host-derived miRNAs secreted into the intestinal lumen could influence the structure and function of gut microbiota. Thus, host miRNAs mediate a bidirectional gut microbiota-host axis. Further studies are required to elucidate the bigger picture of this complex crosstalk.

Author contributions

K.S. and F.O. conceived and designed the experiments and wrote the manuscript. M. Yamaguchi conducted most of the experiments, collected data, and analyzed data. Y.T., M. Yasui, and E.K. conducted some of the experiments.

Declaration of competing interest

The authors declare no conflicts of interest.

Acknowledgments

The authors would like to acknowledge Dr. Satoru Fukiya for helpful discussions. This work was partly supported by JSPS KAKENHI Grant Numbers 20H02927 and 23H02152.

References

[1] V. Ambros, The functions of animal microRNAs, Nature 431(2004) 350-355. https://doi.org/10.1038/nature02871.

[2] D.P. Bartel, MicroRNAs: genomics, biogenesis, mechanism, and function, Cell 116 (2004) 281-297. https://doi.org/10.1016/s0092-8674(04)00045-5.

[3] M.R. Fabian, N. Sonenberg, The mechanics of miRNA-mediated gene silencing: a look under the hood of miRISC, Nat. Struct. Mol. Biol. 19 (2012) 586-593. https://doi.org/10.1038/nsmb.2296.

[4] N. Malmuthuge, L.L. Guan, Noncoding RNAs: regulatory molecules of host-microbiome crosstalk, Trends Microbiol. 29 (2021) 712-724. https://doi.org/10.1016/j.tim.2020.12.003.

[5] K. Sonoyama, F. Ohsaka, Role of microRNAs in the crosstalk between the gut microbiota and intestinal immune system, Biosci. Microbiota Food Health 42 (2023) 222-228. https://doi.org/10.12938/bmfh.2023-027.

[6] S. Liu, A.P. da Cunha, R.M. Rezende, *et al*., The host shapes the gut microbiota via fecal microRNA. Cell Host Microbe 19 (2016) 32-43. https://doi.org/10.1016/j.chom.2015.12.005.

[7] D.B. Sutherland, K. Suzuki, S. Fagarasan, Fostering of advanced mutualism with gut microbiota by Immunoglobulin A, Immunol. Rev. 270 (2016) 20-31. https://doi.org/10.1111/imr.12384.

[8] K. Nakamura, N. Sakuragi, A. Takakuwa, *et al*., Paneth cell α-defensins and enteric microbiota in health and disease, Biosci. Microbiota Food Health 35 (2016) 57-67. https://doi.org/10.12938/bmfh.2015-019.

[9] P. Van den Abbeele, B. Taminiau, I. Pinheiro, *et al*., Arabinoxylo-oligosaccharides and inulin impact inter-individual variation on microbial metabolism and composition, which immunomodulates human cells, J. Agric. Food Chem. 66 (2018) 1121-1130. https://doi.org/10.1021/acs.jafc.7b04611.

[10] S. Hoshi, T. Sakata, K. Mikuni, *et al.*, Galactosylsucrose and xylosylfructoside alter digestive tract size and concentrations of cecal organic acids in rats fed diets containing cholesterol and cholic acid, J. Nutr. 124 (1994) 52–60. https://doi.org/10.1093/jn/124.1.52.

[11] T. Udomsopagit, A. Miwa, M. Seki, *et al*., Intestinal microbiota transplantation reveals the role of microbiota in dietary regulation of RegIIIβ and RegIIIγ expression in mouse intestine, Biochem. Biophys. Res. Commun. 529 (2020) 64–69. https://doi.org/10.1016/j.bbrc.2020.05.150.

[12] E. Bolyen, J.R. Rideout, M.R. Dillon, *et al*., Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2, Nat. Biotechnol. 37 (2019) 852–857. https://doi.org/10.1038/s41587-019-0252-6.

[13] B.J. Callahan, P.J. McMurdie, M.J. Rosen, *et al*., DADA2: High-resolution sample inference from Illumina amplicon data, Nat. Methods 13 (2016) 581–583. https://doi.org/10.1038/nmeth.3869.

[14] C. Quast, E. Pruesse, P. Yilmaz, *et al*., The SILVA ribosomal RNA gene database project: improved data processing and web-based tools, Nucleic Acids Res. 41 (2013) D590-6. https://doi.org/10.1093/nar/gks1219.

[15] N. Segata, J. Izard, L. Waldron, *et al*., Metagenomic biomarker discovery and explanation, Genome Biol. 12 (2011) R60. https://doi.org/10.1186/gb-2011-12-6-r60.

[16] S. Mandal, W. Van Treuren, R.A. White, *et al*., Analysis of composition of microbiomes: a novel method for studying microbial composition, Microb. Ecol. Health Dis. 26 (2015) 27663. https://doi.org/10.3402/mehd.v26.27663.

[17] Y. Xie, L. Yao, X. Yu, *et al*., Action mechanisms and research methods of tRNA-derived small RNAs, Signal Transduct. Target. Ther. 5 (2020) 109. https://doi.org/10.1038/s41392-020-00217-4.

[18] S. Liu, R.M. Rezende, T.G. Moreira, *et al*., Oral administration of miR-30d from feces of MS patients suppresses MS-like symptoms in mice by expanding *Akkermansia muciniphila*, Cell Host Microbe 26 (2019) 779-794. https://doi.org/10.1016/j.chom.2019.10.008.

[19] Y. Liu, H.C-H. Lau, J. Yu, Microbial metabolites in colorectal tumorigenesis and cancer therapy, Gut Microbes 15 (2023) 2203968. https://doi.org/10.1080/19490976.2023.2203968.

[20] B. Krawczyk, P. Wityk, M. Gałęcka, *et al*., The many faces of *Enterococcus* spp.-commensal, probiotic and opportunistic pathogen, Microorganisms 9 (2021) 1900.

https://doi.org/10.3390/microorganisms9091900.

Figure legends

Figure 1 Effect of murine fecal small RNA supplementation on the growth and fermentation of cultured murine fecal bacteria. *A* shows the experimental design, graphs in *B*, *C*, and *D* show OD600 values, pH values, and SCFA concentrations in the culture, respectively. Panel *E* shows representative micrographs of cultured fecal bacteria supplemented with Cy3-labeled fecal small RNAs. Results are presented as standard box plots showing medians and interquartile ranges with minima and maxima values (panels B-D), n=6 per group. Mean values between groups were compared using Dunnett's T3 multiple comparison test followed by Brown-Forsythe one-way ANOVA. Results not sharing common letters are significantly different ($P < 0.05$).

Figure 2 Effect of murine fecal small RNA supplementation on the structure of cultured murine fecal bacteria. Graphs *A* and *B* show the Chao1 and Shannon α-diversity indexes, respectively. Graphs *C* and *D* show PCoA plots of the β-diversity computed based on the unweighted and weighted UniFrac distances, respectively. *E* and *F* show the comparison of relative taxonomic abundance at the genus level analyzed by LEfSe and ANCOM, respectively. Graphs *G-I* show the total number of bacteria, *Enterococcus* spp., and the ratio of *Enterococcus* spp., respectively. In graphs *A, B, G, H,* and *I*, results are presented as standard box plots composed of medians and interquartile ranges with minima and maxima, n=5 and 6 in the vehicle and small RNA-supplemented groups, respectively. Mean values between groups were compared using Welch's *t*-test ($P < 0.05$). ns, not significant.

Figure 3 Effect of murine fecal small RNAs fractionated by ultrafiltration on the increase of *Enterococcus* ratio. Graphs *A*, *B*, and *C* show the size distribution of unfractionated fecal small RNAs, concentrate, and filtrate of ultrafiltration, respectively. Graph *D* shows the increase in *Enterococcus* ratio in the cultured fecal microbiota supplemented with unfractionated fecal small RNAs, concentrate, and filtrate of ultrafiltration. In graph *D*, results are presented as fold induction relative to the vehicle-supplemented culture and shown as standard box plots composed of medians and interquartile ranges with minima and maxima values, n=6 in each group. Mean values between groups were compared using Dunnett's T3

multiple comparison test followed by Brown-Forsythe one-way ANOVA. Results not sharing common letters are significantly different ($P < 0.05$).

Figure 4 Comparison of ability to increase *Enterococcus* between fecal small RNAs and negative control miRNA. Graphs *A*, *B*, and *C* show the total number of bacteria, *Enterococcus* spp., and the ratio of *Enterococcus* spp., respectively. Graphs *D* and *E* show pH values and SCFA concentrations in the culture, respectively. Results are presented as standard box plots composed of medians and interquartile ranges with minima and maxima values, n=6 in each group. Mean values between groups were compared using Dunnett's T3 multiple comparison test followed by Brown-Forsythe one-way ANOVA. Results not sharing common letters are significantly different ($P < 0.05$). ns, not significant.

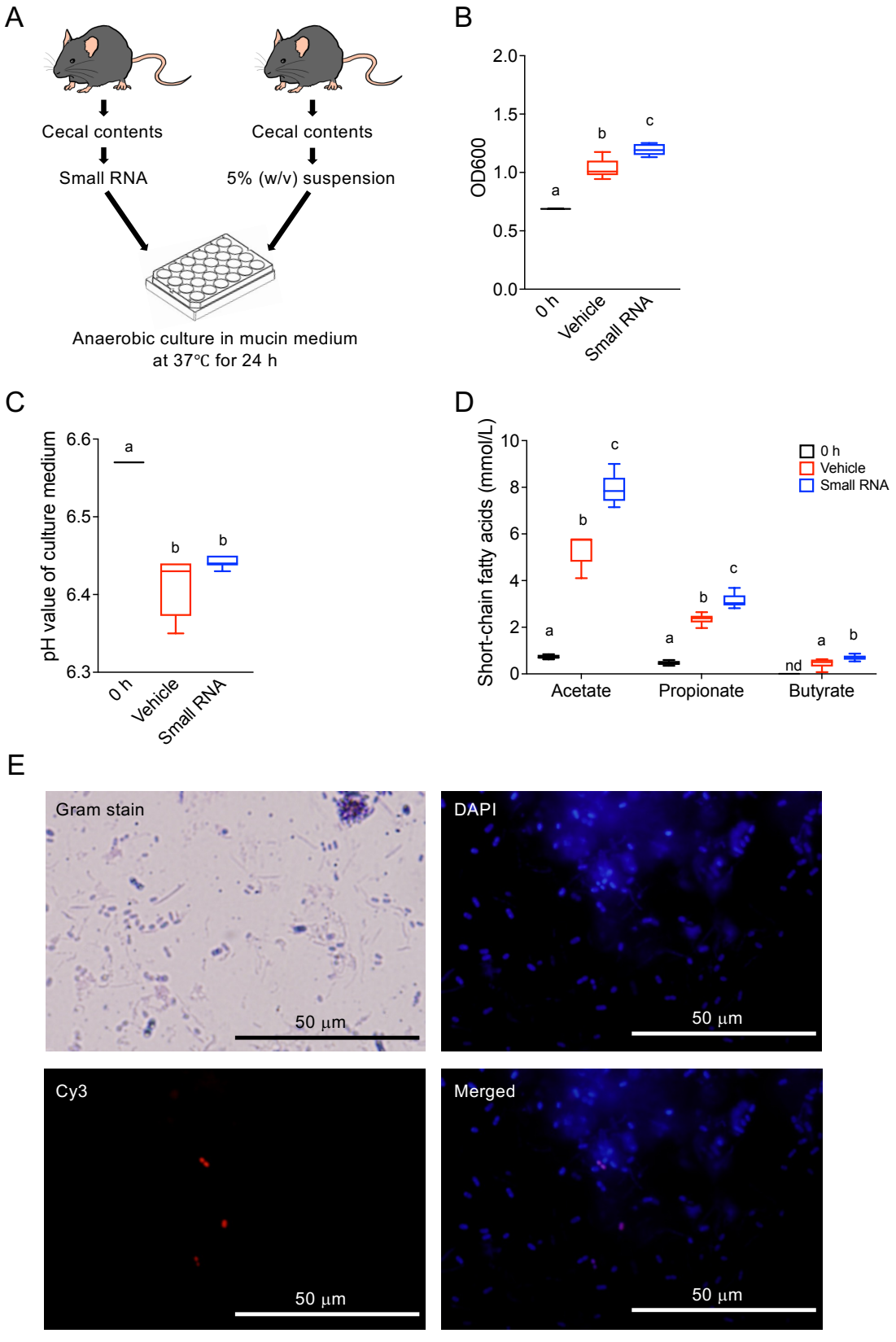


Fig. 1 Ohsaka *et al.*

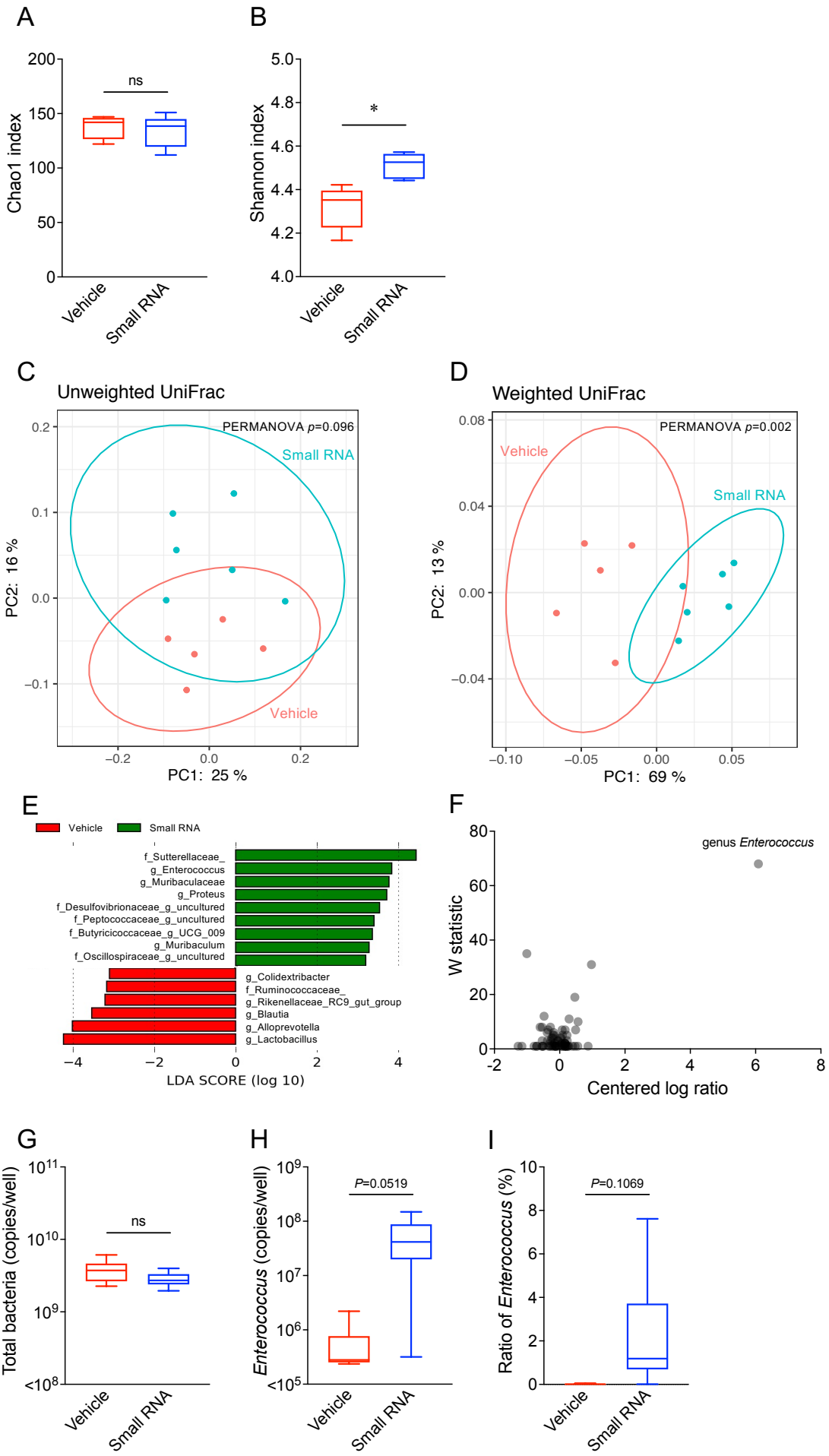


Fig. 2 Ohsaka *et al.*

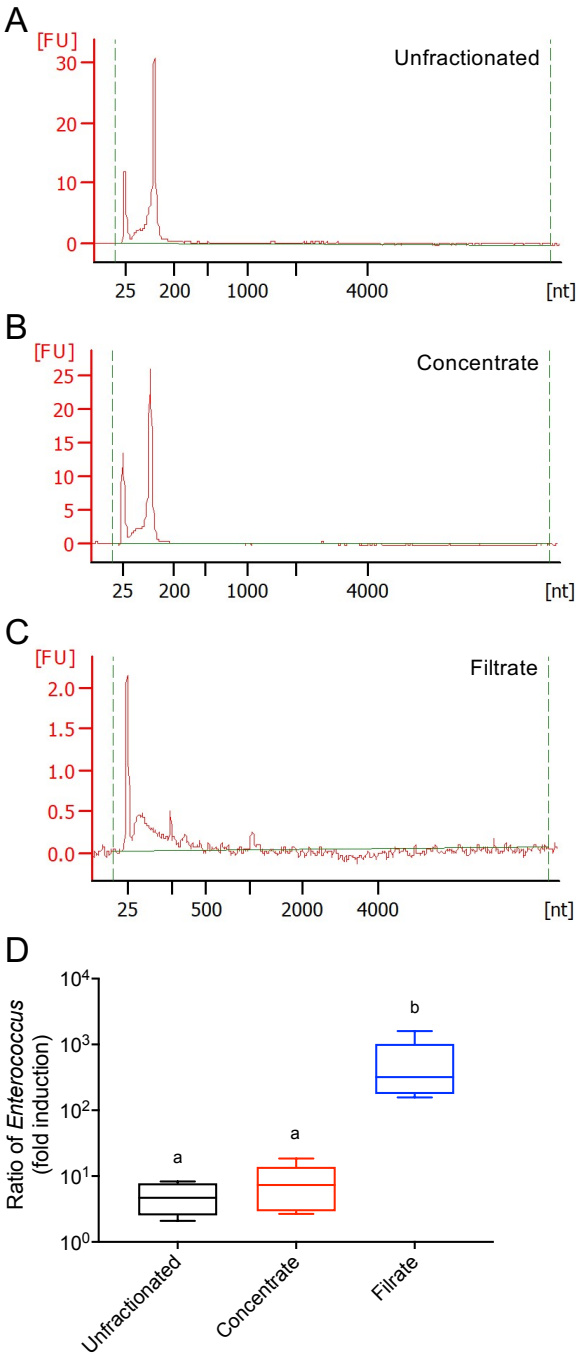


Fig. 3 Ohsaka *et al.*

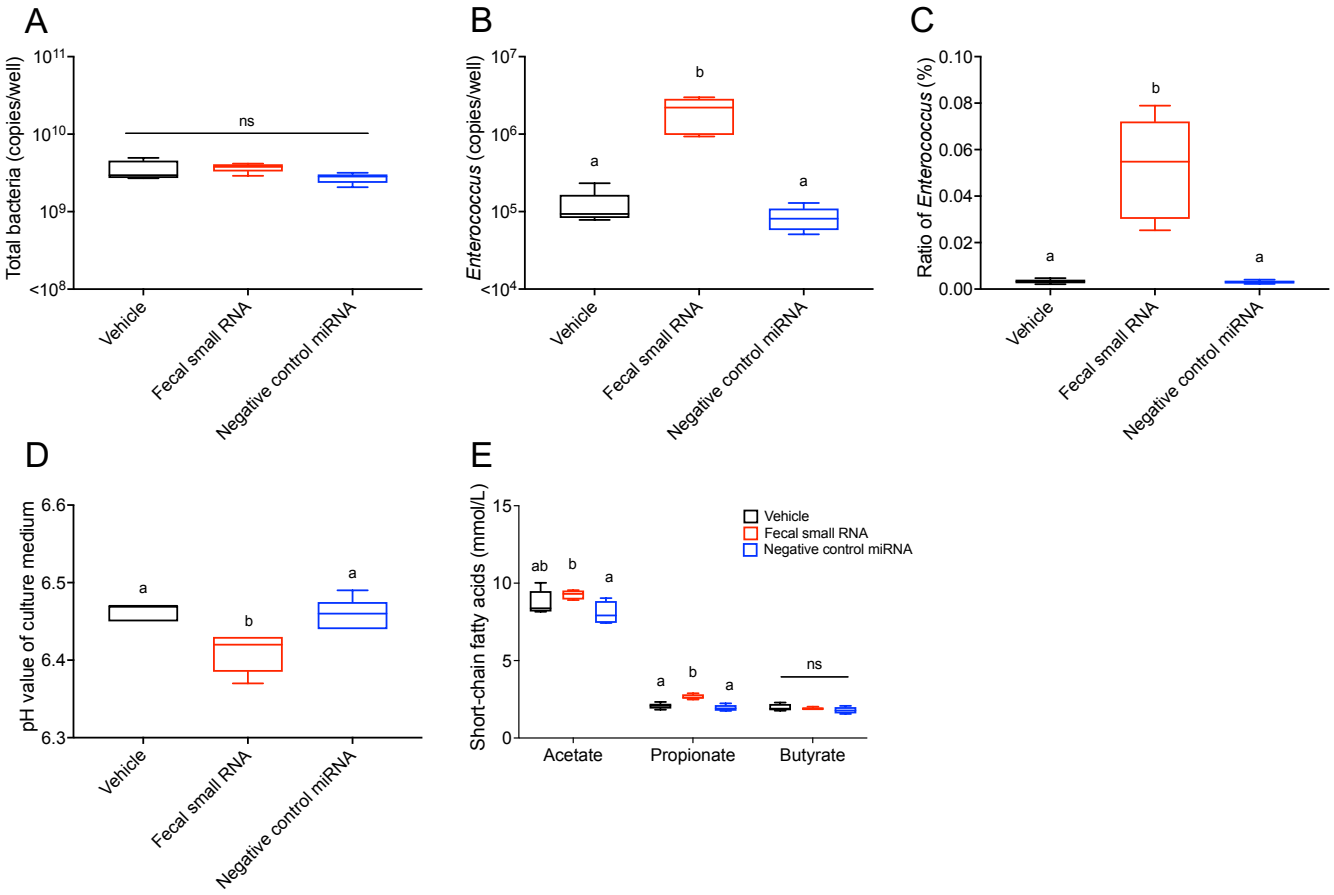


Fig. 4 Ohsaka *et al.*