Fermentation ability of gut microbiota of wild Japanese macaques in the highland and lowland Yakushima: *in vitro* fermentation assay and genetic analyses

Goro Hanya[1], Janko Tackmann[2], Akiko Sawada[1,3], Lee Wanyi[1], Sanjeeta Sharma Pokharel[4], Valdevino Gisele de Castro Maciel[5], Akito Toge[1], Kota Kuroki[1], Ryoma Otsuka[6], Ryoma Mabuchi[7], Jie Liu[8], Masaomi Hatakeyama[9,10], Eri Yamasaki[9], Christian von Mering[2], Rie Shimizu-Inatsugi[9], Takashi Hayakawa[1,11,12], Kentaro K. Shimizu[9,13], Kazunari Ushida[3]

[1] Primate Research Institute, Kyoto University, Inuyama, Japan
[2] Institute of Molecular Life Sciences, University of Zurich, Zurich, Switzerland
[3] Chubu University Academy of Emerging Sciences, Kasugai, Japan
[4] Centre for Ecological Sciences, Indian Institute of Science, India
[5] Amazon National Institute of Research, Manaus, Brazil
[6] Graduate School of Asian and African Area Studies, Kyoto University, Kyoto, Japan
[7] Graduate School of Science, Kyoto University, Kyoto, Japan
[8] Wildlife Research Center, Kyoto University, Kyoto, Japan
[9] Department of Evolutionary Biology and Environmental Studies, University of Zurich, Zurich, Switzerland
[10] Functional Genomics Center Zurich, Zurich, Switzerland
[11] Faculty of Environmental Earth Science, Hokkaido University, Sapporo, Japan
[12] Japan Monkey Centre, Inuyama, Japan
[13] Kihara Institute for Biological Research, Yokohama City University, Yokohama,

Japan

Correspondence to: G. Hanya: E-mail: hanya.goro.5z@ kyoto-u.ac.jp, ORCID iD: https://orcid.org/0000-0002-8612-659X

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Author Contributions

Goro Hanya, Akiko Sawada, Christian von Mering, Rie Shimizu-Inatsugi, Kentaro K. Shimizu, and Kazunari Ushida designed the research. Goro Hanya, Akiko Sawada, Sanjeeta Sharma Pokharel, Valdevino Gisele de Castro Maciel, Akito Toge, Kota Kuroki, Ryoma Otsuka, Ryoma Mabuchi, Jie Liu, Takashi Hayakawa and Kazunari Ushida collected samples and perfomed the *in vitro* fermentation assay. Goro Hanya, Akiko Sawada, Lee Wanyi, Eri Yamasaki, Rie Shimizu-Inatsugi, and Takashi Hayakawa conducted the genetic analyses, Janko Tackmann and Masaomi Hatakeyama analyzed the sequence data. Goro Hanya wrote the manuscript.

Abstract

Wild Japanese macaques (*Macaca fuscata* Blyth) living in the highland and lowland areas of Yakushima are known to have different diets, with highland individuals consuming more leaves. We aim to clarify whether and how these differences in diet are also reflected by gut microbial composition and fermentation ability. Therefore, we conduct an *in vitro* fermentation assay using fresh feces from macaques as inoculum and dry leaf powder of *Eurya japonica* Thunb. as a substrate. Fermentation activity was higher for feces collected in the highland, as evidenced by higher gas and butyric acid production and lower pH. Genetic analysis indicated separation of highland and lowland in terms of both community structure and function of the gut microbiota. Comparison of feces and suspension after fermentation indicated that the community structure changed during fermentation, and the change was larger for lowland samples. Analysis of the 16S rRNA V3-V4 barcoding region of the gut microbiota showed that community structure was clearly clustered between the two areas. Furthermore, metagenomic analysis indicated separation by gene and pathway abundance patterns. Two pathways (glycogen biosynthesis I and D-galacturonate degradation I) were enriched in lowland samples, possibly related to the fruit-eating lifestyle in the lowland. Overall, we demonstrated that the more leaf-eating highland Japanese macaques harbor gut microbiota with higher leaf fermentation ability compared to the more fruit-eating lowland ones. Broad, non-specific taxonomic and functional gut microbiome differences suggest that this pattern may be driven by a complex interplay between many taxa and pathways rather than single functional traits.

Keywords: diet, *in vitro* fermentation assay, meta-16S analysis, digestion, generalists

Introduction

There are three steps in feeding—searching, processing and digestion—and animals adapt in various ways to maximize nutritional intake and/or minimize intake of toxic compounds at each step. Many animal traits, including locomotion [1], morphology [2], vision [3], gustation [4], and digestion enzymes [5] are, at least partly, a result of this adaptation process, and some of these traits are genetically fixed. While specialization to a particular food type opens up normally inaccessible resources [6], such specialization may, on the other hand, limit overall resource range [7]. Since environmental fluctuations can occur at much shorter time scales compared to adaptive evolution, a generalist strategy may require sufficient flexibility to respond to rapidly changing food conditions [8]. Flexibility can, for instance, be achieved by behavioral adaptation, including changes in activity budget [9], ranging [10], and feeding techniques [11], which have been intensively studied among large-brained, behaviorally flexible generalist animals such as primates [12]. These studies mainly focused on the question of how such behavioral adjustments can increase net food intake.

Recently, as another class of feeding-related flexible adaptations, much attention has been given to the role of the gut microbiome. A typical human individual harbors 10-100 trillion symbiotic microorganisms, the majority of which are gut microbes [13]. Human gut microorganisms are estimated to possess 3.3 million non-redundant genes [14], compared to only around 22,000 genes found in the human genome [15]. Host and microbial symbiont genomes often have

complementary roles in digestion [16]. Cellulose, xylan, and many other polysaccharides are fermented by gut microbes to produce short-chain fatty acids (SCFA), an important energy source for vertebrates [17]. In fact, energy gain through SCFAs is estimated to reach as high as 10% in humans [18] and 31% in a non-human primate species [19]. Furthermore, the genomic diversity of gut microbes among different hosts is much larger than that for the hosts themselves [13], which allows gut microbiota to respond more quickly to environmental fluctuations than their hosts. Another function of the gut microbe is detoxification, i.e. degradation of a plant's toxic and anti-nutritional compounds [20, 21].

Enabled by the development of next-generation sequencing techniques, a rapidly increasing number of studies on the flexibility of the gut microbiome have been published [22]. Many of these studies sequence hypervariable regions of the microbial 16S rRNA gene to assess the taxonomic composition of any given gut community. Community composition can change flexibly with regard to habitat [23-25], age and sex of the host [26, 27], social contact with other animals [28], and season [29-31]. Observational studies of wild populations [32] as well as feeding experiments on captive animals [33] show that the gut microbiome composition adjusts flexibly in response to changes in diet. However, data on community composition is often difficult to link directly to function because many functional genes, including those involved in digestion, are shared across diverse microbial groups [34]. As a result, even though the feeding behavior of non-human primates has been studied in detail, there are few studies, as described below, that clarified the digestive effectiveness of the gut microbiota of wild animals in a direct, straightforward experimental setting.

One way to reveal the specific gut microbiome adaptations is to study the presence and/or expression patterns of functional genes through metagenomic and metatranscriptomic analyses [16, 35]. Combined with metabolomic analysis [36], this approach can enable researchers to track the metabolic pathways involved in digesting specific foods into absorbable nutrients, thus providing a deeper understanding of the role of gut microbes in these processes at the molecular level. However, each food contains various nutrients and toxins, and numerous microbial species with large numbers of genes are involved in their digestion. Data obtained by these omics approaches are thus immense and complex; consequently, extracting system-level insights on the interplay between hosts and their microbial symbionts can be a considerable challenge [34].

Another way to answer an ecologically important, simple question on the effectiveness of the gut microbe in food digestion is *in vitro* fermentation assay [37]. In such assays, live gut microbes contained in fresh feces of animals are used as inoculum with a specific food as a substrate, which is subsequently fermented under *in vitro* conditions (e.g. anaerobic and 37˚C). If one type of microbial community is more efficient at fermenting the substrate than others, the overall fermentation activity, such as gas or SCFA production, is expected to be larger. While this method is an established practice in the area of animal husbandry, it is rarely used in wildlife studies. Indeed, previous work on non-human primates has so far been mostly limited to zoo animals [38-41], with the exception of a single study revealing the fermentation ability of plant gum by the gut microbiota of wild chimpanzees [42]. These studies on captive animals found that the relationship between diet and fermentation ability was complex

and non-specific. However, gut microbiome composition can be significantly modified under captivity [43, 44], and recent studies also highlighted the importance of study *in natura*, or in natural environments [45]. Thus, applying *in vitro* fermentation to wild animals *in natura* provides a unique opportunity to study microbiome-related fermentation responses under natural conditions.

The subject of this study, the Japanese macaque (*Macaca fuscata* Blyth), is an ideal species for studying the digestive role of the gut microbiome in a fluctuating environment, since they modify their diet in response to regional and seasonal variations in food availability [46, 47]. In our study site, Yakushima, there is an elevational gradient in fruit production [48]: while the main foods in the fruit-poor highland are fiber-rich foods, such as mature leaves [49], diet in the fruit-rich lowland features more fruits and seeds [50]. These two areas are only about 7 km apart and genetic variation between macaque populations is generally low [51]. Therefore, non-genetic, flexible adjustments are required to survive under the differing food conditions provided by these two contrasting habitats. Since macaques are habituated to human presence in many places in Yakushima [52], the collection of fresh feces is feasible. To further minimize disturbance of the fecal microbiota, *in vitro* fermentation assays can be performed directly on-site at a research station of Kyoto University.

In this study, we examined the hypothesis that the gut microbiome of a generalist host is shaped by the host's flexible feeding behavior and contributes to digestion in a food-type-specific manner. In particular, we tested the prediction that the gut microbiome of the more leaf-eating highland macaques is more capable of fermenting leaves than that of the more fruit-eating lowland macaques. We evaluated the fermentation ability by quantifying gas and SCFA

187 production in an *in vitro* fermentation assay. Furthermore, we conducted genetic
188 analyses of the fecal samples used in the fermentation assay to better
189 understand which microbial taxa and genes might be responsible for the
190 observed differences in fermentation ability. To this end, we first conducted a
191 meta-16S rRNA gene analysis to determine gut microbiome community profiles
192 in both the highland and lowland, which allowed us to examine whether
193 community composition significantly differs between regions and to identify
194 differentially abundant taxa for each region. We also compared between feces
195 and corresponding suspension samples after fermentation to reveal the changes
196 in the community structure during the assay. Subsequently, we conducted a
197 complementary whole-genome shotgun analysis of the fecal samples to
198 additionally examine functional differences (i.e. in terms of gene and pathway
199 composition). Apart from investigating broad functional differences, we also
200 specifically compared the abundance of 37 genes known to be involved in
201 fermentation. These genes participate in most upstream and downstream stages
202 of the synthesis of SCFAs from polysaccharides.
203
204 Methods
205 Study sites
206 Yakushima is an island in southern Japan (30 ˚N, 131 ˚E) with an area of 503
207 km^2, with the highest peak being 1936 m in elevation. In the lowland, warm
208 temperate evergreen broad-leaved trees, such as *Castanopsis cuspidata*,
209 *Quercus salicina*, and *Distylium racemosum*, mix with subtropical plants. In the
210 highland, warm-temperate evergreen broad-leaved trees, such as *Q. acuta*, *Q.*
211 *salicina*, and *D. racemosum*, are interspersed with conifers (including

212 *Cryptomeria japonica*, *Abies firma*, and *Tsuga sieboldii*). Further details of the
213 study site are described in Hanya et al. [48].
214
215 Study subjects
216 The subjects of this study, Japanese macaques, have a home range of ca 0.5–1
217 km^2 [53] and their daily path length is 2.2–2.6 km [54]. The collection sites in the
218 highland and lowland were more than 3 km apart from each other, so the two
219 sites were used by different groups. In May, when we collected samples for this
220 study, the main foods were reported to be sapfruits of *Myrica rubra* in the lowland
221 and mature leaves of *Symplocos myrtacea* and *Eurya japonica* in the highland
222 [49, 50], which was consistent with our *ad libitum* observation at the time of
223 collection. Neutral detergent fiber content is higher for leaves of *S. myrtaceae*
224 (34.1%) and *E. japonica* (50.8%) than the pulp of *M. rubra* (22.4%) [55].
225
226 Sample collection
227 On May 22-24, 2016, we collected fresh feces of wild Japanese macaques in the
228 eastern and western highland (730-1330 m a.s.l.; N=12), as well as the western
229 lowland (150-190 m a.s.l.; N=15), of Yakushima (Fig. 1). Defecation was directly
230 observed for the majority of samples, and in all other cases, feces were judged
231 to be fresh (<1 h defecation) because a monkey group was still in the proximity
232 and the surface of the feces showed moistness. During sampling, we first
233 collected a small amount of fecal materials with a cotton swab and kept it in a 2
234 mL tube filled with RNAlater [56]. To avoid environmental contamination, these
235 genetic samples were collected only from the inner part of the feces, which did
236 not touch the soil. One sample was collected for genetic analysis but could not

be included as an inoculum of the subsequent fermentation assay. We then put all remaining fecal material in a sealed plastic bag, which was semi-vacuumized using a kitchen vacuumizer. The plastic bags were labelled and kept in a jar filled with blue ice. We recorded the sampling locations with a GPS device (GPSMAP 60CSx ®Garmin) and brought the samples to the field station of the Wildlife Research Center of Kyoto University in Nagata village within four hours of sample collection.

In vitro fermentation assay

In the laboratory, one part of the fecal samples was mixed with four parts of McDougall's buffer (ingredients (g/L): $NaHCO_3$ (9.8), $Na_2HPO_4 \cdot 2H_2O$ (2.44), KCl (0.57), NaCl (0.47), $MgSO_4 \cdot 7H_2O$ (0.12), $CaCl_2 \cdot 2H_2O$ (0.16)) which was pre-heated at 39˚C and saturated with CO_2 gas. We confirmed the pH of the buffer before use as 6.8. We squeezed the mixture through two layers of sterile gauzes to remove large particles in the feces. We subsampled 5 mL of the filtrate, then centrifuged it at 2,600 x g for 5 min at 4˚C. The supernatant was stored at -30°C for subsequent measurement of SCFA at time “zero” of the incubation. However, we mistakenly neglected to collect two samples from highland and those seven from lowland, which were therefore excluded from the SCFA measurement. As a substrate, leaves of *Eurya japonica* Thunb. were previously collected in Inuyama city, central Japan. We chose this species because the macaques in both highland and lowland eat this [49]. The leaves were dried at 40˚C and then ground by a mill (Wonder Blender WB-1 ®Osaka Chemical) before use. A portion of the filtrate (100 ml) and one gram dry weight of powdered *E. japonica* leaves were poured into a serum bottle. Then, the bottles

were closed with butyl rubber stoppers and aluminum seals after replacing the head-space gas with 100% CO_2. Bottles were placed in a water bath at 37˚C with continuous shaking for 24 h. Gas production, an indicator of fermentation activity, was measured at 6 h, 12 h, 18 h and 24 h of incubation by displacing a plunger of a 10-ml glass syringe in a manner similar to the method established by Blümmel and Ørskov [57]. After 24 h, the pH of inoculum was measured and additional samples were collected for DNA analysis of the suspension after fermentation (SAF, hereafter). Then, it was centrifuged at 2,600 x g for 5 min at room temperature, and the supernatant was collected for SCFA analysis.

SCFA analysis

Analysis of SCFA was conducted by TechnoPro R&D, Inc., Nagoya, Japan. The samples were centrifuged at 10,000 rpm for 5 min at 4°C, and the supernatant was filtered with 0.45 μm PVDFfilter (Millex-HV filter, Merk Millipore). The filtrates were diluted with the same volume of ethanol. Carboxylic acids were pre-labelled with 2-nitrophenylhydrazine using the Short- and Long-Chain Fatty Acid Analysis Kit (YMC, Co. Ltd, Kyoto, Japan). The SCFA derivatives were subsequently extracted by n-hexane and diethyl ether and finally evaporated to a dry state. The residue was dissolved in methanol and filtered through a 0.45-μm membrane filter. A portion (10 μl) of filtrate was injected into an HPLC system with YMC-Pack FA column (250 × 6.0 mm; YMC Co., Ltd.). We applied the isocratic elution mode with the acetonitrile/methanol/water mobile phase (30:16:54, v/v) at 50°C. Six SCFAs (acetic, propionic, butyric, isobutyric, valeric and isovaleric acids) were measured for absorbance at 400 nm. The sum of individual SCFA concentrations was regarded as total SCFA.

287

288 Genetic analysis

289 Approximately one week after collecting the samples, we centrifuged the sample
290 tubes at 14000 rpm for 10 min and discarded the supernatant (RNAlater
291 solution). This was carried at Primate Research Institute, Kyoto University,
292 Japan. The pelleted samples were then frozen at -80°C until extraction, except
293 at the time of export to Switzerland on September 29 (31 hours, temperature <=
294 4◦C at all times). Further analysis was conducted at the Department of
295 Evolutionary Biology and Environmental Studies, University of Zurich, and the
296 Functional Genomics Center Zurich. We added four zirconia beads (3 mm in
297 diameter), 0.1 mg zirconia/silica beads (0.1 mm in diameter), and 500 µL of
298 Inhibit Ex buffer of QIAamp Fast DNA Stool Mini Kit ® QIAGEN into the tubes
299 and crushed the sample substrate with a bead crusher at 1800 rpm for 5 min.
300 DNA was extracted by QIAamp Fast DNA Stool Mini Kit following the
301 manufacturer's protocol. We quantified the DNA concentration with Qubit dsDNA
302 HS Assay Kit and a Qubit fluorometer ®Thermo Fisher Scientific.
303 We conducted meta-16S analysis for both feces and SAF samples. We
304 amplified the V3-V4 region of the 16S rRNA gene with the following primer set:
305 S-D-Bact-0341-b-S-17 (forward) CCT ACG GGN GGC WGC AG and
306 S-D-Bact-0785-a-A-21 (reverse) GAC TAC HVG GGT ATC TAA TCC [58]. We
307 chose this region because it has already been used for a previous study of
308 Japanese macaques and it can represent the composition of gut microbe
309 community as well as other regions, such as V1-V2 [56]. We purified the PCR
310 amplicons using Agencourt AMPure XP beads ®Beckman Coulter, Inc. and then
311 performed a second PCR (using the Illumina Nextera XT Index Kit) to attach

312 specific dual indices and sequencing adapters to each amplicon. DNA
313 concentration of the 2nd PCR products were quantified and pooled so that the
314 molarities of all samples were equalized. We evaluated the fragment size
315 distribution with TapeStation ®Agilent Technologies, Inc. After Phi X spike-in, the
316 library was sequenced on the Illumina Miseq platform (2 x 300 bp). Further
317 details of the protocol are described in Hayakawa et al. [56].
318 We conducted the shotgun metagenomics analysis for only feces
319 samples. We fragmented the DNA with Covaris E220 ®M&S Instruments, Inc.,
320 following the manufacturer's recommended setting to make the average DNA
321 size 200 bp (http://covarisinc.com/wp-content/uploads/pn_010308.pdf). After
322 confirming the fragment size by TapeStation, we prepared a library using Ultra™
323 II DNA Library Prep for Illumina ®New England Biolabs Inc. and sequenced it
324 with the Illumina Hiseq 4000 platform (2 x 150 bp).
325
326 Data analysis
327 The difference in gas and SCFA production between the highland and lowland
328 were tested by t-test using R 3.2.2.
329 For the 16S rRNA gene analysis, demultiplexing yielded 7,424,940
330 paired-end reads for downstream analysis (median/minimum per sample:
331 244,875/78,467). Reads were denoised with dada2 (version 1.12.1, [59]) using
332 read truncation lengths of '280, 250', maximum expected errors of 8, pooling
333 option 'pseudo,' maximum allowed mismatches when merging pairs of 3, and
334 default values for all other options. Denoising resulted in 25,518 Amplicon
335 Sequencing Variants (ASVs), which were subsequently aligned using
336 INFERNAL (version 1.1.2, [60]) with the microbial secondary structure model

SSU-ALIGN. Based on this alignment, ASVs were further clustered into 2,541 *de novo* OTUs (97% sequence identity) using HPC-CLUST (version 1.2.1, [61]) with the average linkage option. Taxonomic classification was performed by mapping all ASVs with MAPseq (version 1.2.1, [62]) against its default reference database (MAPref 2.2b, see [63] for details on how it was generated). ASV-specific classifications were then summarized into consensus classifications per OTU by retaining only classifications with >90% assigned representatives (MAPseq confidence cutoff >= 0.5).

For phylogenetic tree construction, representative ASVs with the highest abundance were chosen for each OTU. Representatives were aligned using INFERNAL (see above), and the resulting alignment was subsequently used to construct a phylogenetic tree with FastTree (version 2.1.9, [64]) using the GTR substitution model and otherwise default options. Alpha- and Beta-diversity indices were computed using the python package scikit-bio (version 0.5.1, http://scikit-bio.org/), and standard statistical tests (Student's t-test for independent samples, Mann-Whitney U test) were performed with the python package scipy (version 0.19.1; https://www.scipy.org/). Differential abundance analysis on all OTUs and taxa found in at least five samples was conducted using edgeR (version 3.24.3, [65]) with robust dispersion (function estimate GLMRobustDisp) and normalization factor option TMM.

For the shotgun metagenomics analysis, de-multiplexing yielded 395,051,334 paired-end reads for downstream analysis. Read pairs were joined and filtered using mothur (version 1.38.1, [66]) using the "make.contigs" and "screen.seqs" methods (the latter with options maxambig 10, maxhomop 10, maxlength 480), which resulted in 373,262,340 filtered reads (5.5% removed).

To remove putative eukaryotic sequences, all filtered reads were mapped against KEGG (release 59, [67]) with the DIAMOND tool (version 0.8.38, [68], options “sensitive” and “-b 20”) and all reads with a eukaryotic best hit were discarded (16,091,001, or 4.3%).

Based on all remaining reads, gene and pathway abundances were generated with HUMAnN2 (version 0.11.1, [69]) using the recommended UniRef90 database [70] for gene definitions and the MetaCyc database [71] for pathway definitions (both databases downloaded in February 2018), as well as relative abundance normalization (option “relab”). Level 4 EC identifiers were assigned based on HUMAnN2 utility mapping files. Standard statistical tests were performed using the python package scipy (see above).

Compliance with Ethical Standards

During the fieldwork, we adhered to the “Guideline for field research of non-human primates” of the Primate Research Institute, Kyoto University. Furthermore, our procedure complied with ARRIVE guidelines for the use of animals in research (http://www.nc3rs.org.uk/ARRIVE), as well as the legal requirements of Japan. No prior consent from the Japanese government is required to export biological samples from Japan in the context of Convention on Biological Diversity (http://www.env.go.jp/en/nature/biodiv/abs/index.html).

Results

In vitro fermentation assay

We found the overall fermentation activity to be higher for fecal samples from the highland compared to the lowland, as evident from both significantly higher gas

387 production (6 h, 12 h, 18 h, and 24 h; Fig. 2a) and significantly lower pH (Fig. 2b).
388 There was no significant difference in total SCFA production between the
389 highland and lowland (Fig. 2c). Among the four SCFAs (acetic acid, propionic
390 acid, butyric+isobutyric acids, and valeric+iso valeric acids), only the production
391 of butyric acid (including isobutyric acid) was significantly higher for highland
392 samples compared to lowland (Fig. 3). Prior to fermentation (time point 0 h), we
393 found no significant difference in total SCFA concentration or for any of the four
394 SCFA classes.
395
396 Comparisons of community structure of the fecal microbiota
397 We obtained 7,424,940 reads (median/minimum per sample: 244,875/78,467)
398 which were successfully demultiplexed, filtered and *de novo* clustered into 2541
399 operational taxonomic units (OTUs) using a 97% sequence identity cutoff (see
400 Methods). Out of these OTUs, 1311 (51.6%) were classifiable at the phylum
401 level or deeper. A summary of the phylogenetic assignment of the OTUs in each
402 sample is shown in Supplemental Material 3.
403 The differences in relative abundance of major taxa (>1% at the phylum,
404 class, order, family and genus levels) were all insignificant, except for significant
405 enrichment of Anaeroplasmataceae in the highland (Supplemental Material 3, 4).
406 Differential abundance analysis revealed 130 OTUs that were significantly
407 enriched in highland (54) or lowland (76) samples. Enriched bacterial OTUs in
408 the highlands were 12 OTUs of phylum Firmicutes, class Clostridia, order
409 Clostridiales, one OUT of *Bacteroides uniformis* in the phylum Bacteroidetes,
410 family Bacteroidaceae and the remaining 39 OTUs could not be mapped to any
411 taxa. Enriched OTUs in the lowland included 16 OTUs of Bacteroidetes (all were

412 class Bacteroidia, order Bacteroidales), eight OTUs of Firmicutes (order
413 Clostridia and Negativicutes), two OTU of Proteobacteria, 23 OTUs of
414 Proteobacteria (all were *Treponema berlinense*) and 27 unmapped OTUs.
415 Average alpha diversity did not differ between the lowland and highland
416 as differences in observed OTUs: Chao1, Shannon and Faith's Phylogenetic
417 Diversity indices were all insignificant (Supplemental Material 1).
418 Community structure clearly differed between the highland and lowland,
419 based on pairwise Bray-Curtis dissimilarity (PERMANOVA, P = 0.001). In
420 addition to clustering separately from lowland samples, highland samples
421 furthermore showed increased variability and sub-structure (Fig. 4). This
422 sub-structure could not be explained by longitudinal differences, since eastern
423 and western samples did not cluster separately.
424
425 Comparisons of community structure between the feces and suspension after
426 fermentation (SAF)
427 Community structure of the SAF was significantly different from the
428 corresponding fecal samples (Fig. 4, PERMANOVA, highland: pseudo-F=1.86,
429 P=0.022; lowland: pseudo-F=3.06, P=0.001). Judging from the F and P values,
430 the difference in community structure between SAF and feces in the lowland was
431 larger than that in the highland.
432 The number of taxa showing significant changes in abundance
433 between SAF and feces was larger in the lowland (three phyla, four classes, six
434 orders, eight families, two genera and 104 OTUs) than in the highland (one
435 phylum, no class, one order, two families, one genus and 47 OTUs) (Table 1).
436 Among the major taxa (>1% of relative abundance in feces or SAF), significantly

437 more enrichment in SAF than in feces was detected for Proteobacteria (highland
438 and lowland) and Spirochaetes at the phylum level and Streptococcaceae
439 (highland and lowland), Succinivibrionaceae (lowland), and Pasteurellaceae
440 (lowland) at the family level and *Streptococcus* (highland and lowland) at the
441 genus level.
442 None of the alpha diversity indices differed significantly between feces
443 and SAF in either the highland or the lowland (Supplemental Material 1).
444
445 Metagenomic analysis of the fecal microbiota
446 After having assessed the community composition, we next focused on
447 systematic and specific functional (gene and pathway) differences between
448 highland and lowland microbiota. To this end, we conducted whole-genome
449 shotgun sequencing on all fecal samples, which yielded 373,262,340 reads, or
450 14,356,243 reads/sample, after de-multiplexing and filtering. PERMANOVA
451 analysis (based on pairwise Bray-Curtis dissimilarity) showed a statistically
452 significant separation between lowland and highland samples in terms of both
453 gene abundance ($P = 0.001$; Fig. 5a) and pathway abundance ($P = 0.034$; Fig.
454 5b). In contrast to abundance, pathway coverage showed no significant
455 lowland-highland separation ($P = 0.11$; Fig. 5c).
456 Among 37 selected genes involved in the metabolism of
457 polysaccharides and the synthesis of SCFAs from pyruvate, the abundance was
458 not significantly different for any of the genes.
459 When mapping the presence and abs243ence of the selected genes on
460 polysaccharide metabolism and SCFA synthesis pathways, most genes were
461 uniformly present or uniformly absent in both the lowland and highland samples

462 (Supplemental Material 2). Regarding polysaccharide metabolism, most gut
463 microbiota possessed genes to catalyze cellulose, xylan, mannan, and pectin
464 degradation, all of which are lacking in the genome of rhesus macaques (the
465 most closely related species of Japanese macaques with a published genome).
466 Pathways for the synthesis of acetate, butyrate, and propionate from pyruvate
467 were all inter-connected for most fecal metagenomes, both in the lowland and
468 highland, and also within the macaque reference genome.
469 Out of 263 tested MetaCyc [71] pathways (present in > 10 samples),
470 two showed a significant difference in abundance between highland and lowland
471 samples: GLYCOGENSYNTH-PWY (glycogen biosynthesis I, from
472 ADP-D-Glucose); Mann-Whitney U test, P = 0.041, Benjamini-Hochberg
473 adjusted) and GALACTUROCAT-PWY (D-galacturonate degradation I;
474 Mann-Whitney U test, P = 0.044, Benjamini-Hochberg adjusted), both of which
475 were more abundant in lowland.
476
477 Discussion
478 Difference in fermentation ability between highland and lowland
479 The *in vitro* fermentation assay clearly indicated that the fermentation ability of
480 leaves was higher for highland gut microbiota compared to lowland communities.
481 In the highland, where the macaque diet is leaf-based, the corresponding
482 communities produced significantly more gas and induced a significantly larger
483 decrease in pH during fermentation. While they also showed a trend toward
484 increased SCFA production, this difference was not significant. We note,
485 however, that this may be caused by power issues due to decreased sample
486 size compared to the gas and pH measurements (nine samples were not

487 included, see Methods). SCFAs are utilized as an energy source by the host,
488 and they constitutes a considerable portion in both human and non-human
489 primates' energy intake [18, 19]. Taken together, our results indicate that the
490 higher leaf-fermentation potential of highland gut communities may facilitate
491 digestion of the leaf-based diet of highland macaques, compared to the lowland
492 animals. While more research is necessary, this finding highlights the potential
493 importance of the gut microbiome for generalist hosts (such as the macaques
494 studied here) to flexibly respond to and benefit from changing food conditions.
495 Interference experiments on humans show that dietary changes can modify the
496 gut microbiome within only a few days (2-5) [72], so the gut microbiota of
497 macaques in the highland and lowland, living separately for years (in the case of
498 philopatric females) or months (in the case of immigrant males), likely have
499 sufficient transition time to respond to different dietary regimes.

500 The assay also suggested that the fermentation system was different
501 between the two areas. When investigating individual classes of SCFAs, we
502 found butyric acid production to be significantly increased in highland compared
503 to lowland samples. Many bacteria are capable of acetic acid production, but
504 propionic or butyric acid producers are generally less common [34]. Furthermore,
505 biosynthesis pathways for butyric acid are highly diverse and can often vary
506 even within a single bacterial family [34]. Some butyrate producers can produce
507 butyrate with the aid of other bacteria. For example, lactate is produced by other
508 species, and the resultant lactate is converted to butyrate by lactate users, such
509 as *Megasphaera* [73]. Increased butyric acid production has been reported in
510 many different contexts: for example, butyric acid in feces has been reported to
511 be higher when energy intake decreased in howler monkeys [31], while butyric

512 acid production increased with higher fruit consumption in gorillas [74]. For
513 human hosts, the situation is generally mixed, since some interference
514 experiments in which additional fiber was added to the normal diet led to
515 increased fecal butyrate concentration, while other studies did not report such
516 increases [75]. Our finding of increased butyric acid production in highland
517 microbiota (exposed to leaf-based diets) contributes to this growing body of
518 research and, while the overall picture is still complex, provides additional
519 evidence for a link between high-fiber diets and the production of this SCFA.
520
521 Meta-16S analysis: Gut microbiota community structure
522 Gut community structure was clearly clustered between the highland and
523 lowland populations in Yakushima. A previous study using the meta-16S analysis
524 of wild and captive Japanese macaques included samples collected in the
525 highland and lowland Yakushima (N=18), which also indicated clear
526 differentiation of gut microbe community structure between the two areas [44]
527 (Supplemental Materials 5). We did not directly compare those results with the
528 current study because the methods of that previous study were not the same as
529 the method used in the current work, nevertheless that study provided further
530 confirmation of the validity of our findings. While we observed higher OTU profile
531 variation and considerable sub-structure within highland samples compared to
532 lowland ones, this sub-clustering did not correspond to longitudinal differences in
533 sampling locations (i.e. east vs. west). This is interesting because western
534 highland macaques live in the immediate geographical vicinity of the lowland
535 population (in contrast to the eastern highland population), but this co-locality is
536 not reflected by a higher similarity in the gut microbe community. It thus seems

537 that habitat similarity, rather than physical contact between hosts (typically via
538 male immigration and emigration) [76], is responsible for shaping the gut
539 communities. Unmeasured factors related to group identity may provide an
540 alternative explanation for the sub-structure within highland samples, but more
541 data would be needed for definite conclusions.

542 Comparisons between feces and suspension after fermentation (SAF)
543 indicated that the community structure of the microbiota was significantly altered
544 during fermentation than it used to be in the gut. This alteration seems to be
545 larger for lowland than highland, evidenced by the F and P statistics and the
546 larger number of taxa showing changes in abundance. This suggests that the
547 difference between the experimental and original conditions in the gut was larger
548 for lowland than for highland microbiota, which is consistent with the fruit-based
549 diet in the lowland.

550 The comparisons of differential abundance between the feces and SAF
551 may give us a clue to the bacterial taxa that are responsible for the fermentation
552 activity. For example, Succinivibrionaceae (phylum Proteobacteria, class
553 Gammaproteobacteria, order Aeromonadales) increased during fermentation.
554 This family is reported to increase in the feces of wild Tibetan macaques during
555 winter, when they eat more leaves [29]. Interestingly, however, in the rumen of
556 cows, this family increased when the animals are supplied with low-fiber diet [77].
557 Another example is *Streptococcus* (phylum Firmicutes, class Bacilli, order
558 Lactobacillales, family Streptococcaceae). This genus includes the species with
559 tanning-binding ability [20, 21], which may be useful for digesting leaves. Feces
560 of feral pigs with high feed efficiency, and thus with a higher ability to degrade
561 cellulose, contain more *Streptococcus* [78]. These studies are consistent with

562 our results that this genus increased during the assay using mature leaves as a
563 substrate. However, in wild howler monkeys, this genus increased when they ate
564 low-fiber foods (fruits) [31]. The Succinivibrionaceae and *Streptococcus*
565 examples show that the same taxa may exhibit a different function in
566 fermentation in different hosts. We note, however, that even one species of
567 microorganisms has a wide variety of genes and thus metabolic potential [79]
568 and, therefore, we can expect the deviations at the higher taxonomic levels.
569 Furthermore, it has been shown for different environments that stable community
570 function can be retained even under high species turnover [80, 81]. For the same
571 trait—especially if it features many species and pathways, such as in fiber
572 digestion—taxonomic composition may therefore differ widely. Our results
573 further underscore this notion and caution against generalized assumptions on
574 community function that are based solely on taxonomy.
575 In contrast to a recent study on fecal microbiota of the same host
576 species [56], a large fraction of OTUs (48.4%) could not be taxonomically
577 classified even at the phylum level in our analysis. This difference is due to the
578 more precise mapping approach we used here (MAPseq; [62]), which assigns
579 low confidence to a read if multiple taxa have similar alignment scores and can
580 thus not be confidently distinguished. In various benchmarks, this approach was
581 shown to yield better classifications than the commonly used less conservative
582 alternatives [62, 82]. The high fraction of largely unclassified OTUs may be an
583 indication that much is still to be learned about the macaque gut microbiome.
584
585 Metagenomic analysis: Difference with respect to the genes and pathway
586 composition

Metagenomic analysis indicated that highland and lowland populations also differed on the level of genes and pathways. This shows that the function of the gut microbe varies between the two areas, in which macaques experience different food and thermal conditions [46, 83].

For example, we found two pathways that differed in abundance between highland and lowland samples, which may be related to the lifestyle of the host animals. For example, the glycogen biosynthesis I pathway was more abundant in the lowland samples. Glycogen is synthesized to store excess glucose temporarily in the liver [84]. The main food in the sampled season in the lowland of Yakushima is the sapfruit of *Myrica rubra* [85], which is considered one of the most sugar-rich fruits available in Yakushima [86]. Therefore, lowland macaques may experience situations of having excess glucose more often than leaf-eating highland macaques. Genes in this pathway could thus be beneficial for storing excess glucose and using it as an energy source in the subsequent food-scarce season. In contrast, in the highland, saving the function of the pathway may be advantageous when the macaques do not eat fruits. The D-galacturonate degradation I pathway was also enriched in the lowland; D-galacturonate is an oxidized form of galactose, and it is included in many polysaccharides in plants, including pectin [87]. This pathway may also be related to the fruit-dominated diet in the lowland, but this remains unknown without detailed knowledge of the metabolomics profile of their foods.

We did not find a genetic difference which can directly account for the observed differences in fermentation ability between highland and lowland. Among the 37 genes encoding enzymes that catalyze the majority of upstream and downstream reactions in the degradation of polysaccharides to SCFAs, we

found no difference in enrichment patterns for any of the genes. It was evident that the gut microbiota in both regions possess genes necessary for the synthesis of SCFAs from polysaccharides but are lacking in the host genome (assuming it is the same as that of rhesus macaques). Although not as important as in the highland, leaves are also one of the main foods in the lowland [50], and all of these genes may be necessary for survival in both areas.

Implications of the flexibility in digestive ability

In this study, we demonstrated that the gut microbiome of macaques is shaped according to habitat and diet, in terms of both community structure (taxa, OTUs) and the function (genes, pathways). Furthermore, we showed that these differences affect the ability to digest leaves and thus may play an important adaptive role in this generalist host. Since similar alterations in the gut microbial community composition in response to dietary changes are a well-known fact in many hosts [22], we predict similar microbiome-mediated increases in digestive ability for commonly consumed foods in many other generalist animals.

We can point out a number of limitations in the interpretations of the current study. The inoculum of this assay is feces, not gut contents. Even though fecal samples are usually used as a representative of ‘gut’ (colon) microbiota, these microbiota are subject to changes immediately after defecation due to biotic and abiotic factors [88]. We found no difference in the fecal SCFA, or 0 h concentrations between the highland and lowland. It is possible that this was simply due to the smaller sample size than gas production reduced by mistake, but it is also possible that the difference was a real one. Fecal SCFA concentration is determined not only by the microbiota but also by the digesta,

637 which are derived from many different kinds of foods in the case of wild animals.
638 Some bacteria in feces in the lowland may have worked to produce as much
639 SCFAs *in vivo* as in the highland in the presence of natural foods, but not when
640 supplied with only leaves of *E. japonica*. Therefore, different substrates should
641 be tested in the future to confirm our conclusion.
642 The above limitations of the current *in vitro* fermentation assay suggest
643 that further improvement of this method is possible as a way to reveal the
644 fermentation ability of the gut microbe. In this study, we examined only one type
645 of substrate, but it is possible to divide the fecal samples into multiple portions
646 and use these as an inoculum for different substrate types. Similarly, we
647 investigated leaf-fermentation ability only for one plant species eaten frequently
648 in the highland but only rarely in the lowland; consequently, it would also be
649 interesting to test the fermentation of substrates commonly eaten in the lowland,
650 since these may be processed more efficiently by microbial communities from
651 lowland hosts. In our assay, the fermentation time was set to 24 h, which
652 approximates the typical retention time in the hindgut of Japanese macaques
653 (total gut retention time can be up to 35 h when feeding on high-fiber foods; [89]).
654 However, retention time can vary based on different factors, including the body
655 size of the animal, and this should be taken into consideration in future studies.
656 Furthermore, our time-series data on gas production suggest that the
657 fermentation process is time-dependent, so it may be possible to mimic the *in*
658 *vivo* digestion process much better via a more dynamic and flexible experimental
659 setup, including substrate preparation and the duration of fermentation. In future
660 studies, it will also be necessary to evaluate the relative contribution of the
661 fermentation by the gut microbiome compared to the enzymatic digestion by the

host. This is necessary to better understand how gut microbial shifts can affect the digestion and survival of the host. Another interesting avenue of study would be to complement the metagenomics data used here with approaches based on metabolomics [36, 74] and metatranscriptomics [16, 90] approaches, which could provide deeper insights into the molecular basis and mechanisms affecting the fermentation potential. It would also be interesting to isolate bacteria species from the feces and to explore its function, for example, by sequencing the whole genome. We have already isolated and sequenced the genome of *Sarcina ventriculi* from the feces of Japanese macaques in the highland [91]. The genome includes potentially important genes for the digestion of leaves, such as cyanate metabolism. Interestingly, this bacteria cannot be isolated from feces collected in the lowland. Using combinations of these multi-level approaches would clarify the mechanism that produces different fermentation abilities among the host animals living in different environments.

In conclusion, we demonstrated that gut microbe community structure differed considerably between the two investigated habitats, which may in part be explained by habitat-specific diets. In support of this, via an *in vitro* fermentation assay, we showed that gut microbiota of the more leaf-eating highland Japanese macaques have a higher fermentation ability for leaves than those of the more fruit-eating lowland animals. Taken together, our results indicate that gut microbiota may help generalist hosts to improve their digestive ability in response to the variations on food availability.

Conflict of Interest: The authors declare that they have no conflict of interest.

687 Ethical approval: During the fieldwork, we adhered to the “Guideline for field
688 research of non-human primates” of the Primate Research Institute, Kyoto
689 University. According to the guideline, our study is purely non-invasive and does
690 not need approval from an ethical committee.
691
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1037 Draft Genome Sequences of Sarcina ventriculi Strains Isolated from Wild
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1043 Legends for figures

1044 Fig. 1. Map of the study site and location of sample collection. Open squares are
1045 highland samples and filled squares are lowland samples. Note that several
1046 locations are too close to be distinguished on the map. Contours are drawn
1047 every 300 m.

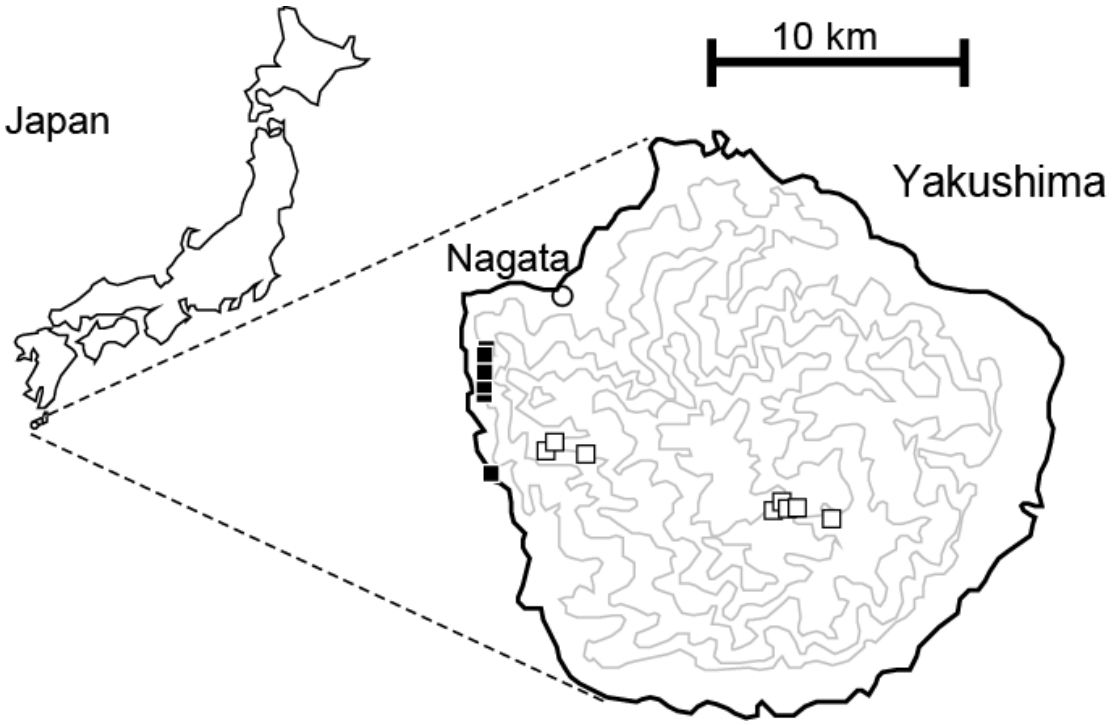
1048 Fig. 2. Difference in fermentation activity between the highland and lowland. (a)
1049 Gas production, (b) pH values after fermentation, and (c) total short-chained
1050 fatty acid (SCFA) production.

1051 Fig. 3. Profile of short-chained fatty acids produced during fermentation.

1052 Fig. 4. Beta-diversity of the community composition of the feces and suspension
1053 after fermentation (SAF) samples in the highland and lowland based on
1054 Bray-Curtis similarity index.

1055 Fig. 5. Beta-diversity of (a) abundance patterns of genes, (b) abundance
1056 patterns of pathway, and (c) coverage patterns of pathway between the
1057 highland and lowland.

1058



1059

1060 Fig. 1

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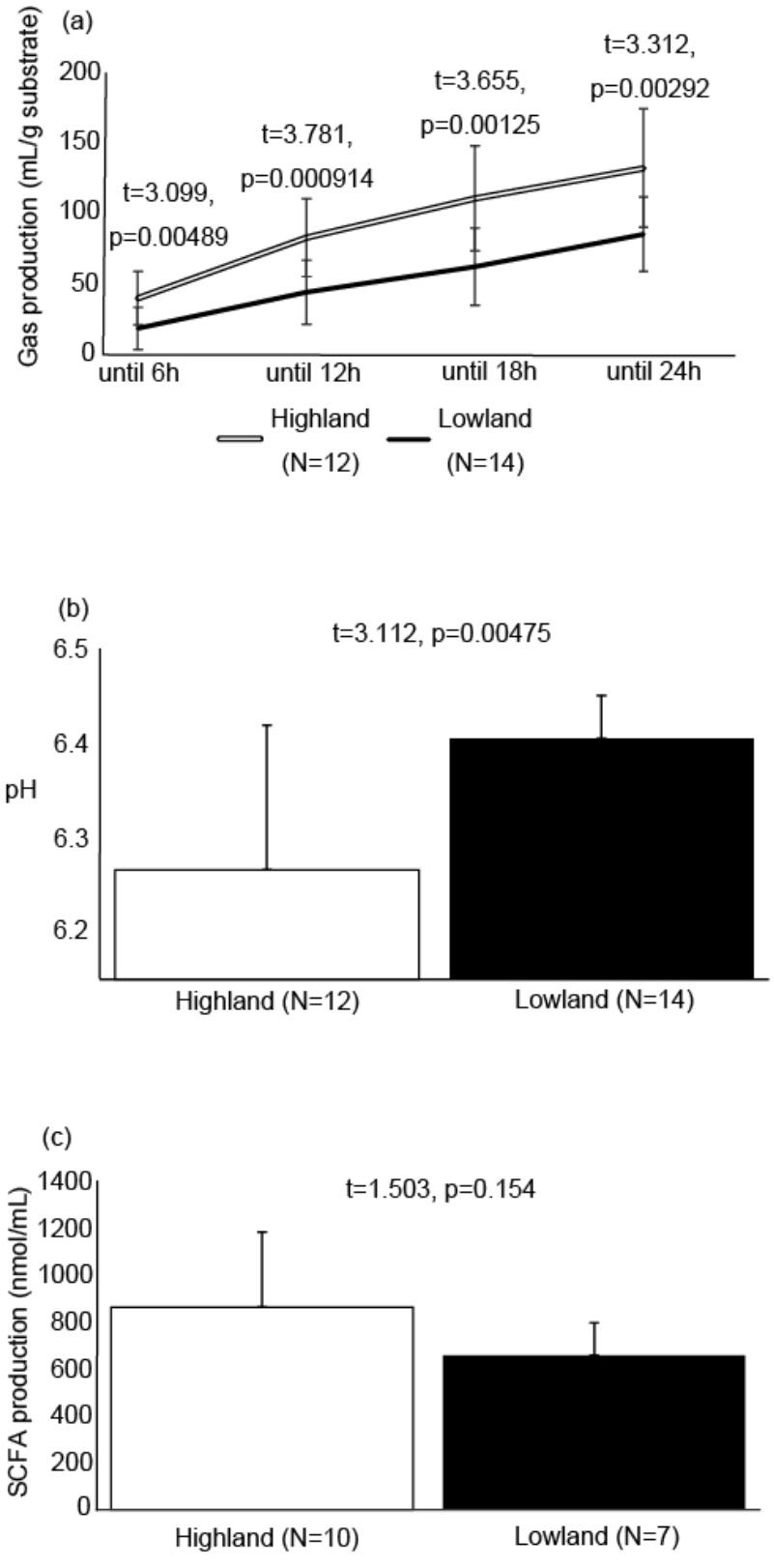

1062

1063 Fig. 2

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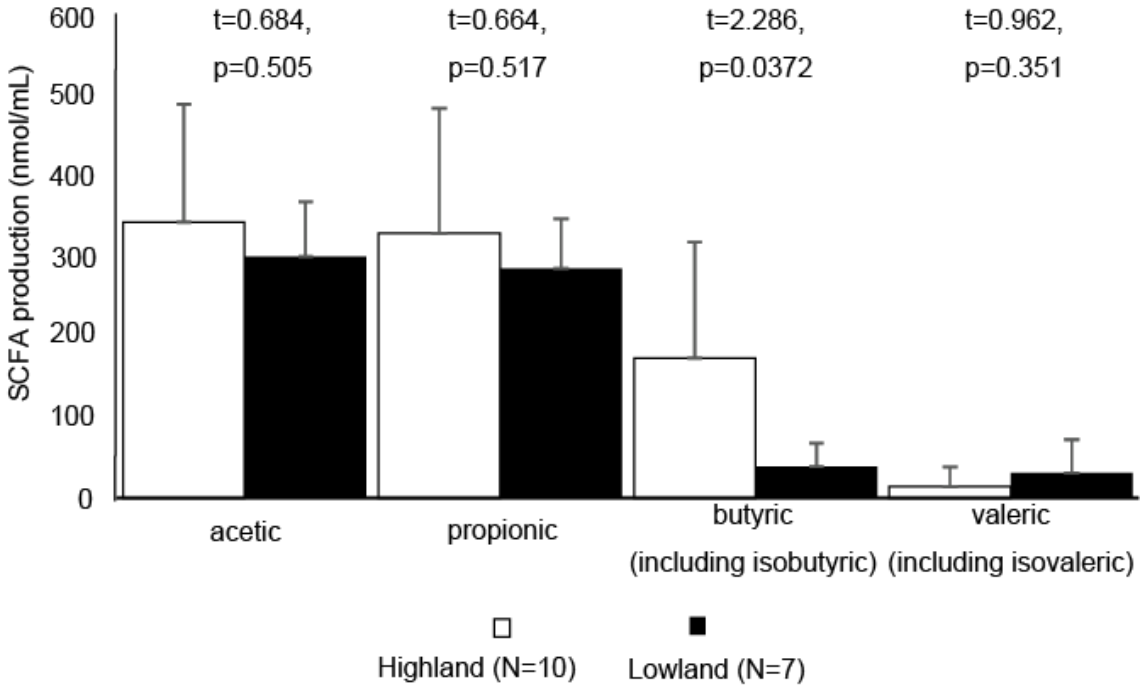

1065

1066 Fig. 3

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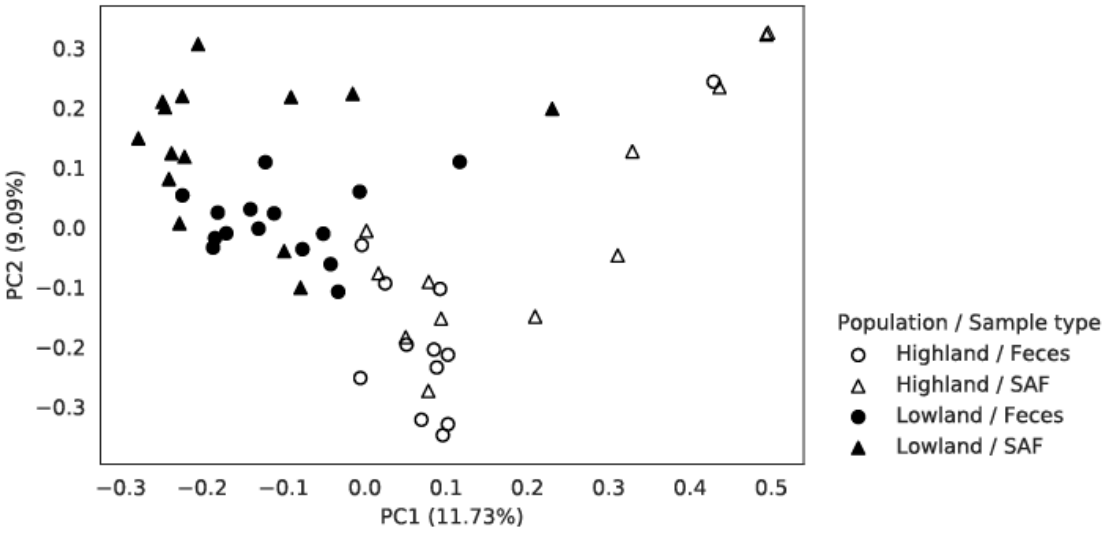

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1070 Fig. 4

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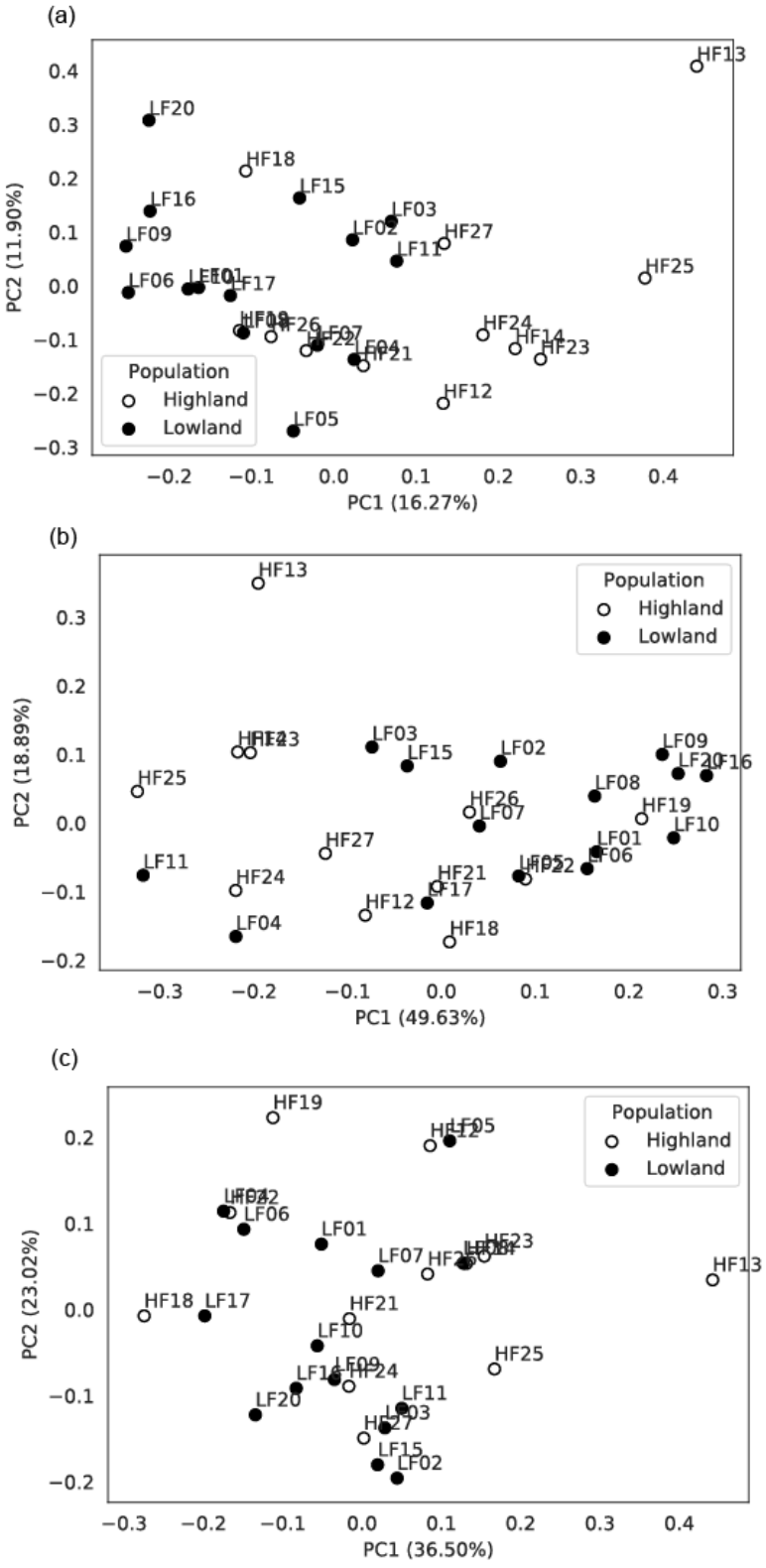

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1074 Fig. 5

Table 1. Significantly enriched taxa in feces or suspension after fermentation (SAF) samples

(a) Highland

Taxonomic level	Taxa	Enriched in	P (after FDR)	Relative abundance	
				Feces	SAF
Phylum	Proteobacteria	SAF	0.0059	0.0686	0.2958
Order	Lactobacillales	SAF	0.0106	0.001	0.0324
Family	Streptococcaceae	SAF	0.0105	0.0009	0.0323
	Comamonadaceae	SAF	0.0112	2E-07	0.0041
Genus	*Streptococcus*	SAF	<0.0001	0.0009	0.0317

(b) Lowland

Taxonomic level	Taxa	Enriched in	P (after FDR)	Relative abundance	
				Feces	SAF
Phylum	Proteobacteria	SAF	0.0004	0.0412	0.1643
	Tenericutes	Feces	0.0159	0.0183	0.0052
	Spirochaetes	SAF	0.0159	0.0625	0.1453
Class	Bacilli	SAF	<0.0001	0	0.0086
	Gammaproteobacteria	SAF	<0.0001	0.0231	0.1552
	Spirochaetia	SAF	<0.0001	0.0625	0.1453
	Negativicutes	SAF	0.0319	0.0104	0.0254
Order	Pasteurellales	SAF	<0.0001	3E-05	0.0336
	Aeromonadales	SAF	<0.0001	0.0168	0.1008
	Lactobacillales	SAF	<0.0001	0	0.0086
	Spirochaetales	SAF	0.0022	0.0624	0.1453
	Acholeplasmatales	Feces	0.006	0.0067	0.0031
	Selenomonadales	SAF	0.0396	0.0025	0.0118
Family	Pasteurellaceae	SAF	<0.0001	3E-05	0.0336
	Streptococcaceae	SAF	<0.0001	0	0.0086
	Oscillospiraceae	SAF	<0.0001	0	0.0029
	Ruminococcaceae	Feces	0.0008	0.0191	0.0073
	Succinivibrionaceae	SAF	0.0015	0.0136	0.0969
	Lachnospiraceae	Feces	0.007	0.0469	0.0167
	Clostridiaceae	Feces	0.0205	0.0016	4E-05
	Anaeroplasmataceae	Feces	0.0308	0.01	0.0017
Genus	*Streptococcus*	SAF	<0.0001	0	0.0086
	Holdemanella	SAF	0.004	1E-05	0.0011