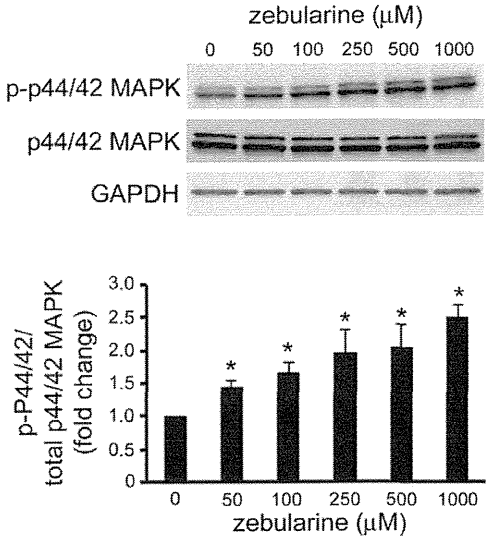

Figure 5. Effects of zebularine on phosphorylation of p44/42 MAPK. The phosphorylation and expression of p44/42 MAPK after zebularine treatment for 24 h at different concentrations. After treatment, the cells were harvested and western blot analysis was performed to detect the phosphorylated and total p44/42 MAPK protein level. GAPDH was used as a loading control. Data are the means ± SEM of results from at least three independent experiments. *$p<0.05$, compared to 0 μM.
doi:10.1371/journal.pone.0054036.g005

(12D1, #2947), p44/42 mitogen-activated protein kinase (MAPK) (137F5, #4695), phospho-p44/42 MAPK (The202/Thy204) (#4370), Bax (D2E11, #5023), Bcl-2 (50E3, #2870), PKR (N216, #2766), DNMT1 (D63A6, #5032) (Cell Signaling Technology Japan, Tokyo, Japan), phospho-PKR (E120, ab32036, abcam, Tokyo, Japan), p53 (M 7001, Dako Japan, Tokyo, Japan), DNMT3a (sc-20703), DNMT3b (sc-81252) (Santa Cruz Biotechnology, Santa Cruz, CA), or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (#MAB374, Millipore, Temecula, CA) antibodies, and then with peroxidase-conjugated secondary antibodies (NA931 or NA940, GE Healthcare Japan, Tokyo, Japan). The bound antibodies were detected using the ECL system (GE Healthcare Japan).

Statistics

All experiments were performed at least three times. Values are expressed as means ± standard error of the mean (SEM). Statistical analyses were performed using an unpaired Student's *t*-test or two-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference as a post-hoc test. $p<0.05$ was considered to indicate statistical significance.

Results

The effects of zebularine on HepG2 cell viability

In order to investigate the effect of zebularine on HepG2 cell viability, we performed WST assay after zebularine exposure. WST assay indicated that zebularine affected cell viability. Exposure of cells to zebularine for 72 h resulted in a decrease in cell viability (Fig. 1A). To further determine whether zebularine could inhibit the proliferation of HepG2 cells, we conducted BrdU incorporation assay after zebularine treatment for 24 h. Although WST assay indicated that zebularine could not affect cell viability after 24 h (Fig. 1B), BrdU incorporation assay clearly showed that the uptake of BrdU by HepG2 cells was already reduced after 24 h exposure to zebularine (Fig. 1C). At a concentration of 250 μM, the uptake of BrdU was reduced to 22.1±0.6% compared with 0 μM and a similar reduction of BrdU uptake (20.1±1.5%) was observed at a concentration of 1000 μM. In addition, we examined whether zebularine could induce HepG2 cell death. Terminal deoxynucleotidyl transferase dUTP nick end labeling

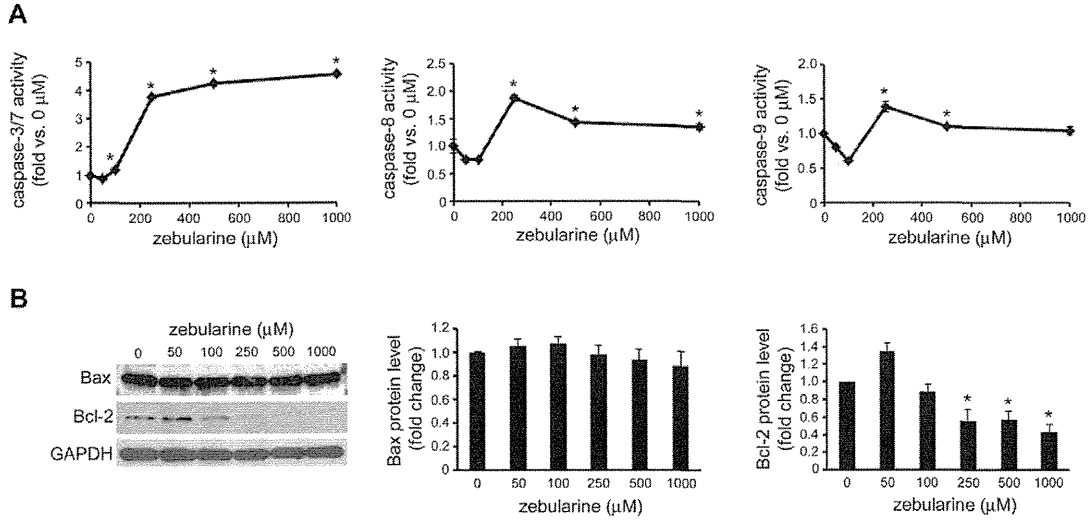

Figure 6. The effect of zebularine on apoptosis-related proteins. HepG2 cells were treated with zebularine at indicated concentrations for 72 h. (A) Caspase-3/7, -8, and -9 activities were determined using Caspase-Glo Assays. The data are expressed as fold-increase relative to the respective untreated samples (RLU/60 min/μg protein). (B) The protein level of Bax and Bcl-2 after zebularine treatment for 72 h at different concentrations. After treatment, the cells were harvested and western blot analysis was performed to detect the protein level of Bax and Bcl-2. GAPDH was used as a loading control. Data are the means ± SEM of results from at least three independent experiments. *$p<0.05$, compared to 0 μM.
doi:10.1371/journal.pone.0054036.g006

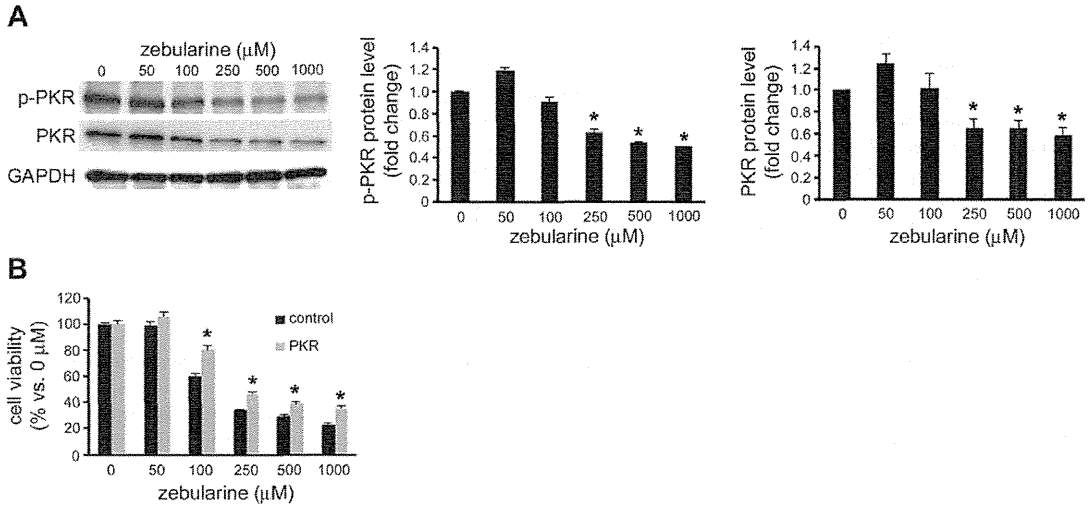

Figure 7. Effects of zebularine on phosphorylation of PKR. (A) The phosphorylation and expression of PKR after zebularine treatment for 72 h at different concentrations. After treatment, the cells were harvested and western blot analysis was performed to detect the phosphorylated and total PKR protein level. GAPDH was used as a loading control. *$p<0.05$, compared to 0 μM. (B) Effect of the overexpression of PKR in zebularine-induced cell death. The forward transfection of the empty vector (Halo Tag control vector) as the control or the plasmid-containing PKR cDNA sequence (pFN21A-hPKR) was performed, and the cells were then treated with different concentrations of zebularine for 72 h. *$p<0.05$, compared to control. Data are the means ± SEM of results from at least three independent experiments.
doi:10.1371/journal.pone.0054036.g007

(TUNEL) assay demonstrated that zebularine induced apoptotic cell death on HepG2 cells. Exposure of cells to zebularine for 72 h resulted in an increase in the number of apoptotic cells (Fig. 1D). These results indicated that DNA replication was blocked and apoptotic cell death was induced by treatment with zebularine, which resulted in reduced HepG2 cell viability.

Zebularine affects HepG2 cells growth arrest and apoptosis via DNA methylation-independent pathway

Because of zebularine's activity as a DNMT inhibitor in other model systems [29,30], its effect on the expression of DNMTs in HepG2 cells was examined. As expected, zebularine treatment was associated with a statistically significant dose-dependent depletion of DNMT1, DNMT3a, and DNMT3b (Fig. 2A).

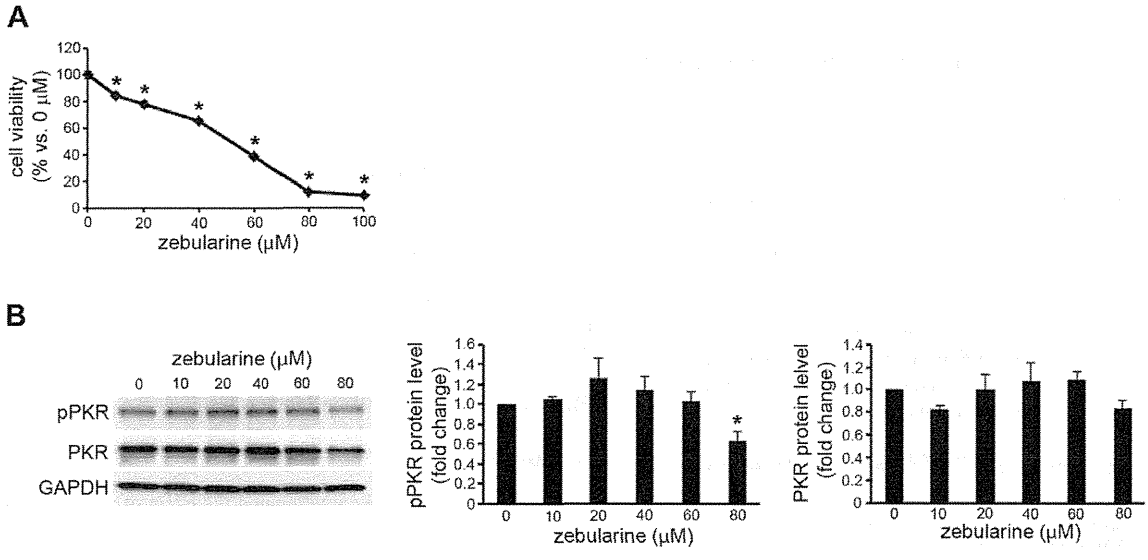

Figure 8. Effects of zebularine on phosphorylation of PKR in HeLa cells. (A) HeLa cells were treated with zebularine at indicated concentrations for 72 h. Cell growth was measured by WST assay. (B) The phosphorylation and expression of PKR after zebularine treatment for 72 h at different concentrations. After treatment, the cells were harvested and western blot analysis was performed to detect the phosphorylated and total PKR protein level. GAPDH was used as a loading control. *$p<0.05$, compared to 0 μM.
doi:10.1371/journal.pone.0054036.g008

Since zebularine decreased DNMT protein levels, to determine whether the growth inhibition and/or apoptosis induction in HepG2 cells by zebularine are a result of a change in DNA methylation, we obtained the genome-wide methylation profiles of zebularine-treated and -untreated (control) HepG2 cells using an Illumina Infinium HumanMethylation450 BeadChip (GEO accession number GSE42490). Among 482,421 assays for CpG sites, 482,260 assays fulfilled our quality control criteria (detection p value <0.01 and no missing beta value for both groups) and were subjected to the following analysis. For each assay, delta-beta value (= average of the beta values of three zebularine-treated samples − average of those of three controls) was calculated. As shown in Fig. 2B, the methylation profiles were highly similar between zebularine-treated and -untreated HepG2 cells. The number of CpG sites whose delta-beta values are >0.1 and <-0.1 was 35 and 162, respectively. At the majority (99.96%) of CpG sites, methylation levels were nearly the same under the two conditions. To further assess whether these minor methylation changes are observed at specific genes or genomic regions, we conducted region-level methylation analysis using the IMA package [28]. Among 26,659 CpG islands (CGIs), only five showed a significant change (adjusted p value <0.05 and $|$delta-beta value$|$ >0.1) of the methylation level upon zebularine treatment (Table S1). All five CGIs were found to be highly methylated in control HepG2 cells (beta value >0.8), and to be partially hypomethylated (delta-beta range -0.11–-0.21) in zebularine-treatment cells. One CGI is located in an intron of the AGAP1 gene that encodes ArfGAP with GTPase domain, ankyrin repeat, and PH domain 1 protein. Another CGI is located 10 kb downstream of the USP18 gene that encodes ubiquitin specific peptidase 18. The other three CGIs are not associated with any RefSeq gene structure (within 50 kb distance). It is unlikely that the slight decrease in DNA methylation at these five CGIs causes growth arrest and apoptosis in HepG2 cells. These results suggest that the administration of zebularine has little effect on DNA methylation in HepG2 cells, and that the inhibited cell growth and induced apoptosis observed in HepG2 cells upon zebularine treatment are caused by unknown mechanisms that are independent of DNA methylation.

Zebularine inhibited CDK and phosphorylation of protein retinoblastoma

To estimate the mechanism by which zebularine inhibits HepG2 cell proliferation, we investigated the change in CDK2 expression that was associated with cell-cycle regulation after zebularine treatment. Our results showed that the levels of CDK2 were downregulated in HepG2 cells at 24 h by zebularine treatment (Fig. 3). Protein retinoblastoma (Rb) plays a critical role in governing cell-cycle progression, especially for the transition from the G1 to the S phase [31], where the total and phosphorylation level of Rb was detected. Our results revealed that phosphorylated Rb (p-Rb) decreased in a concentration-dependent manner 24 h after zebularine treatment, which was accompanied by a reduction in total Rb (Fig. 3).

Zebularine increased $p21^{WAF/CIP1}$ and p53 level in HepG2 cells

Previous studies have demonstrated that tumor suppressor protein $p21^{WAF/CIP1}$ and p53 play an important role in G0/G1 arrest in HepG2 cells [32]. Therefore, in order to determine whether these two proteins play a role in inhibiting cell proliferation, the HepG2 cells were exposed to zebularine and analyzed for change on the protein level of $p21^{WAF/CIP1}$ and p53. The results showed that after 24 h of zebularine treatment, the $p21^{WAF/CIP1}$ and p53 protein level was higher in HepG2 cells than in the control (Fig. 4).

The effect of zebularine on p44/42 MAPK expression

To further clarify the mechanism of the proliferation inhibitory effect of zebularine on HepG2 cells, we examined the expression of p44/42 MAPK in HepG2 cells after zebularine treatment. As shown in Fig, 5, zebularine increased the level of phosphorylated p44/42 MAPK, whereas total p44/42 MAPK was unaffected by the zebularine treatment, as judged by comparisons with GAPDH as a loading control. This data indicates that zebularine can increase the phosphorylation of p44/42 MAPK.

Zebularine induced apoptosis via caspase pathway

To investigate whether zebularine-induced apoptosis was associated with the caspase family proteins, the activity of caspase-3/7, -8, and -9 was examined after zebularine treatment at 72 h. As shown in Fig. 6A, the activity of caspase-3/7 was significantly increased at an apoptosis-inducible concentration of zebularine. In addition to caspase-3, the activity of caspase-8 and -9 was also increased with zebularine treatment. The expression of the proapoptotic factor Bax and the antiapoptotic factor Bcl-2 was examined by western blotting. The result demonstrated that Bax expression was not affected. On the other hand, Bcl-2 expression decreased with an increasing amount of zebularine (Fig. 6B).

Zebularine decreases the activity of PKR in HepG2 cells

A previous study showed that PKR regulates the protein expression level and phosphorylation of Bcl-2 and plays an anti-apoptotic role in HepG2 cells [33]. Since zebularine can reduce the Bcl-2 protein level, we examined PKR and the phosphorylated PKR level with zebularine treatment. Our results showed that zebularine can reduce the phosphorylated PKR level; this was accompanied by a reduction in total PKR (Fig. 7A). To determine whether PKR has an anti-apoptotic effect in HepG2 cells treated with zebularine, we overexpressed the PKR gene in HepG2 cells and exposed the cells to zebularine. We found that zebularine-induced cell death was reduced by overexpression of PKR (Fig. 7B).

The effect of zebularine on the activity of PKR in other cancer cells

Zebularine also inhibits the growth of bladder cancer, breast cancer, and cervical cancer cells [29,30,34]. Since PKR is ubiquitously expressed, we examined whether zebularine decreases the activity of PKR in other cancer cells. It was recently reported that zebularine inhibits the growth of HeLa cervical cancer cells via cell-cycle arrest and caspase-dependent apoptosis [30]. We also observed that zebularine inhibited the growth of HeLa cells, which coincided with the results of the previous study (Fig. 8A). However, our results showed that cell growth inhibiting concentration of zebularine did not reduce the phosphorylated PKR and total PKR levels in HeLa cells (Fig. 8B).

Discussion

In the present study, we investigated the effect of zebularine on human hepatic carcinoma cells and the possible mechanism. To the best of our knowledge, this is the first study to demonstrate that zebularine inhibits hepatic carcinoma cell HepG2 proliferation by inducing cell growth arrest and apoptosis via intrinsic and extrinsic apoptotic pathways.

In this study, we observed that zebularine decreased the level of DNMT1, DNMT3a, and DNMT3b in HepG2 cells. These results

were similar to the reports that DNMT inhibitor induces the depletion of DNMT1, 3a, or 3b protein in human bladder, breast, and cervical cancer cells [24,30,35]. Because tight covalent complexes of zebularine and DNMT could lead to compositional change in DNMT protein, it is plausible that DNMTs can be degraded via a ubiquitination system, consequently being observed in the reduction of its expression [30]. On the other hand, our results suggest that zebularine has little effect on DNA methylation in HepG2 cells. Thus, it seems that the cell-cycle arrest and apoptosis observed in HepG2 cells upon zebularine treatment are caused by mechanisms that are independent of DNA methylation.

Eukaryotic cell proliferation is a highly regulated system that is controlled by CDK-cyclin complexes. The cell-cycle transition from the G1 to the S phase was the major regulatory checkpoint in this process. This transition is characterized by the phosphorylation of Rb, and the CDK-cyclin complex catalyzes the reaction [36,37]. In this study, we found that zebularine inhibited the CDK2 and p-Rb accompanied by a decrease in total Rb, which resulted in cell-cycle arrest and the exertion of its antiproliferative effect. Cell-cycle inhibitor $p21^{WAF/CIP1}$ plays an important role in the G1/S progression process. It may inhibit the activity of the CDK-cyclin complex to regulate cell-cycle progression. These effects can be mediated through p53-dependent or -independent machinery according to the types of stimuli [38–43]. There are two p53-binding elements located at the $p21^{WAF/CIP1}$ gene promoter that can be transactivated by the accumulated nuclear p53 after DNA damage [44]. It is reported that p53-dependent G1 growth arrest is mediated by $p21^{WAF/CIP1}$, and $p21^{WAF/CIP1}$ is the CDK inhibitory protein transcriptionally regulated by p53 [45]. Our results showed that the $p21^{WAF/CIP1}$ level was increased after zebularine treatment. In addition, zebularine also upregulated p53 protein. Thus, in the present study, both p53 and $p21^{WAF/CIP1}$ may perform their function by inhibiting the kinase activities of CDK-cyclin complexes to stimulate cell-cycle arrest, which was attributed to the zebularine effect.

MAPKs are essential components of the intracellular signal transduction pathways that regulate cell proliferation and apoptosis. One subgroup of MAPKs, p44/42 MAPK (ERK1/2), is an important target in the diagnosis and treatment of cancer and has been reported to be required for the upregulation of $p21^{WAF/CIP1}$ that results in cell-cycle arrest [46–48]. Furthermore, the high-intensity p44/42 MAPK signal leads to the repression of CDK2 kinase activity for p-Rb, which mainly regulates the proliferation of HepG2 cells [49]. In the present study, MAPK signaling pathway regulation after zebularine treatments was investigated. We found that zebularine treatment upregulated the phosphorylation of p44/42 MAPK. Therefore, it is suggested that the p44/42 MAPK pathway plays a role in zebularine-induced cell-cycle arrest by regulating the activity of $p21^{WAF/CIP1}$ and Rb.

During the process of apoptosis, caspases are essential for the initiation and execution of cell death in a self-amplifying cascade in response to various stimuli [50]. Two major apoptotic pathways have been identified: the extrinsic and intrinsic apoptotic pathways. The extrinsic pathway is activated by death receptors, which recruit initiator caspase-2, -8, or -10 through adaptor molecules, whereas the intrinsic signals result in the activation of caspase-9. These initiator caspases can sequentially cleave and activate the effector caspase (caspase-3, -6, and -7), which play an important role in mediating cellular destruction [51]. Our results showed that zebularine appeared to induce the apoptosis of HepG2 cells via the intrinsic pathway, as shown by the activation of caspase-9, and the extrinsic pathway, as shown by the activation of caspase-8, which led to caspase-3 activation. Proteins from the Bcl-2 family can be divided into two groups: suppressors of apoptosis (e.g., Bcl-2, Bcl-XL, and Mcl-1) and activators of apoptosis (e.g., Bax, Bok, Hrk, and Bad). These proteins are key regulators of the intrinsic pathway of apoptosis, setting the threshold for engagement into the death machinery [52,53]. Among these, the anti-apoptotic Bcl-2 protein acts to suppress apoptosis by preventing the release of apoptogenic proteins, such as cytochrome c, that reside in the intermembrane space of mitochondria. Functionally, Bax acts in opposition to Bcl-2 and facilitates the release of these mitochondrial apoptogenic factors by translocation and oligomerization [54–56]. Thus, the ratio of Bax/Bcl-2 determines, in part, the susceptibility of cells to death signals and might be a critical factor in a cell's threshold for apoptosis [57]. In this study, the expression of Bax and Bcl-2 proteins in zebularine-treated HepG2 cells was examined by western blot assay. We found that although Bax protein levels were not affected, Bcl-2 protein level was downregulated with zebularine treatment, which led to a marked increase in the Bax/Bcl-2 ratio and then apoptosis.

Initially identified as an antiviral protein, PKR is best known for triggering cell defense responses and initiating innate immune responses by arresting general protein synthesis and inducing apoptosis during virus infection [58]. Activated PKR, known as a eukaryotic initiation factor 2-alpha (eIF-2α) kinase, induces the phosphorylation of eIF-2α [59], which inhibits the initiation of translation through the tRNA-40S ribosomal subunit. On the other hand, PKR is involved in controlling the transcription of Bcl-2 in HepG2 cells, mediated by the transcription factor NF-κB [33]. In this study, we observed that zebularine can reduce the phosphorylation of PKR, which indicates the activated PKR. In addition, overexpression of PKR reduced zebularine-induced cell death. Thus, our results suggest that zebularine decreases the activity of PKR and results in apoptotic cell death via reduced NF-κB activity and the downregulation of Bcl-2. The fact that zebularine inhibits the growth of bladder, breast, and cervical cancer cells [29,30,34] and that PKR is ubiquitously expressed led us to hypothesize that zebularine induced the cell growth arrest via the downregulation of PKR in other cancer cells. When we examined the effect of zebularine on PKR expression in HeLa cells, we observed, however, that zebularine did not decrease the phosphorylation of PKR and the total PKR level. These results suggest that there are differences in the mechanism by which zebularine inhibits cell growth among the different types of carcinomas. The action and mechanisms of zebularine must therefore be further investigated in other cancer cells.

In conclusion, our observation indicated that zebularine inhibited cell growth and induced apoptotic cell death, which contributed to its antiproliferation effects against hepatocellular carcinoma HepG2 cells. The most likely mechanism underlying the zebularine-induced growth arrest involves an initial induction of p44/42 phosphorylation and an increase in $p21^{WAF/CIP1}$ expression, which leads to a reduction in G1-related CDKs such as CDK2 protein and p-Rb, and then ultimately arrests the HepG2 cell cycle. Furthermore, zebularine decreased the activity of PKR, and resulted in apoptotic cell death via the downregulation of Bcl-2.

Supporting Information

Table S1 List of CGIs showing a significant change in DNA methylation level upon zebularine-treatment in HepG2 cells.
(XLS)

Author Contributions

Conceived and designed the experiments: K. Nakamura KH AT. Performed the experiments: K. Nakamura KA K. Nakabayashi NK. Analyzed the data: K. Nakamura K. Nakabayashi KH JY AT. Contributed reagents/materials/analysis tools: JY. Wrote the paper: K. Nakamura K. Nakabayashi AT.

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Robustness of Gut Microbiota of Healthy Adults in Response to Probiotic Intervention Revealed by High-Throughput Pyrosequencing

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Abstract

Probiotics are live microorganisms that potentially confer beneficial outcomes to host by modulating gut microbiota in the intestine. The aim of this study was to comprehensively investigate effects of probiotics on human intestinal microbiota using 454 pyrosequencing of bacterial 16S ribosomal RNA genes with an improved quantitative accuracy for evaluation of the bacterial composition. We obtained 158 faecal samples from 18 healthy adult Japanese who were subjected to intervention with 6 commercially available probiotics containing either *Bifidobacterium* or *Lactobacillus* strains. We then analysed and compared bacterial composition of the faecal samples collected before, during, and after probiotic intervention by Operational taxonomic units (OTUs) and UniFrac distances. The results showed no significant changes in the overall structure of gut microbiota in the samples with and without probiotic administration regardless of groups and types of the probiotics used. We noticed that 32 OTUs (2.7% of all analysed OTUs) assigned to the indigenous species showed a significant increase or decrease of ≥10-fold or a quantity difference in >150 reads on probiotic administration. Such OTUs were found to be individual specific and tend to be unevenly distributed in the subjects. These data, thus, suggest robustness of the gut microbiota composition in healthy adults on probiotic administration.

Key words: probiotics; gut microbiota; 16S ribosomal RNA gene; pyrosequencing

1. Introduction

Probiotics are defined as live bacterial strains conferring various benefits to the consumer by modulating the intestinal ecosystem, thereby potentially promoting host health and improving host disease risk.[1–11] Various probiotic strains have been industrially developed and marketed as a variety of products and applications such as fermented foods and supplements, including yogurt[12–15] Most probiotics taxonomically belong to two genera, *Bifidobacterium* and *Lactobacillus*, that originate from various environments, including the human intestine, and both species are generally regarded as safe.[16–18]

The interaction between administrated probiotics and indigenous microbiota is one of the most attractive and important research areas, particularly because gut microbiota have been shown to be profoundly

associated with various host physiology states, including disease, diet, and age through the shift of bacterial composition, as well as metabolic and nutritional processes.[19–23] The ability of probiotics to survive through the intestine and to modulate gut microbiota is a critical factor in determining their potential for health-related outcomes.

There have been a large number of probiotic intervention studies to assess the impact of probiotics on gut microbiota in healthy adults,[24–34] infants, and children,[35,36] and in clinical trials on patients with a variety of diseases.[37,38] Most probiotic intervention studies were carried out by comparison between probiotic-treated groups and placebo controls and examined only one or two samples from periods before and during intervention or post-intervention for each subject. These experimental designs make evaluation of results obscure from a statistical viewpoint due to the high inter-individual variability of gut microbiota.[4] In addition, most of the analyses focussed on the composition of specific bacterial species or groups by conventional methods such as culturing, quantitative polymerase chain reaction (qPCR), fluorescence *in situ* hybridization, denaturing gradient gel electrophoresis, or terminal-restriction fragment length polymorphism based on the bacterial 16S ribosomal RNA gene (16S). These conventional methodologies may also overlook subtle changes in bacterial community structure and change of species other than targeted species. Thus, the effect of probiotic administration on the overall structure of gut microbiota is largely unknown.

Recently, a high-throughput sequencing-based analysis has been conducted for gut microbiota fed with a probiotic yogurt that provided new insights into probiotics research by utilizing a large-scale dataset.[39] However, much more data are required to understand the impact of probiotics on gut microbiota. Recent advances in sequencing technology have enabled us to elucidate complex bacterial communities, including human gut microbiota.[40,41] Particularly, 454 pyrosequencing of bacterial 16S gene tags coupled with bioinformatics provides a high-throughput and cost-effective approach for the comprehensive analysis of bacterial communities at the species level.[42–48]

In this study, we developed an analysis pipeline for bacterial communities based on barcoded 454 pyrosequencing of 16S gene tags using modified PCR primers that improved the quantitative accuracy of inferred species composition in human gut microbiota. Using this pipeline, we analysed faecal samples longitudinally collected from individuals with and without probiotic administration to evaluate the effect of probiotics on gut microbiota with respect to species richness and diversity. The results revealed the robustness and stability of gut microbiota of healthy adults in response to probiotic administration.

2. Materials and methods

2.1. *Subjects, faecal sample collection, and probiotic intervention*

Eighteen healthy volunteers (age: 22 ± 3.16 yrs, 6 male, 12 female) were recruited through Azabu University, Kanagawa, Japan (Supplementary Table S1). All subjects were informed of the purpose of this study. This study was approved by the ethical committee of Azabu University, and written consent was obtained from all subjects. No subjects were treated with antibiotics during faecal sample collection. The subjects were divided into six groups (three subjects per group), and each group consumed six different commercially available probiotics supplied from Yakult Honsha Co., Ltd, Kagome Co., Ltd, Morinaga Milk Industry Co., Ltd, Takanashi Milk Products Co., Ltd, Meiji Co., Ltd, and Danone Japan Co., Ltd, respectively (Supplementary Table S1). The number of each bacterial strain contained in the probiotic products was estimated as the genome equivalent by qPCR of 16S ribosomal RNA genes using 27Fmod-338R, followed by pyrosequencing of the 16S amplicons (see below). The genome equivalent per gram or millilitre and the total genome equivalent of each bacterial strain in one probiotic product are summarized in Supplementary Table S1. Three subjects in each group consumed the same probiotics daily for 8 weeks according to the schedule of sampling and probiotic intervention (Supplementary Fig. S1). Faecal samples from 4 weeks before (S00) and 8 weeks during probiotic intervention (S01–S04), and 8 weeks after cessation of probiotic intervention (S05–S08), were collected every 2 weeks from each subject. In total, we collected 158 faecal samples from the 18 subjects because we could not collect 1 sample each from 4 of the subjects.

2.2. *Recovery of bacteria from faecal samples*

Freshly collected faeces (1.0 g) were suspended in 20% glycerol (Wako Pure Chemical Industries, Ltd) and phosphate buffered saline solution (Life Technologies Japan, Ltd, Tokyo, Japan), frozen in liquid nitrogen, and stored at −80°C until ready for use. Bacterial pellets were prepared from frozen faecal samples as described previously.[49]

2.3. *DNA isolation from bacteria*

Faecal DNA was isolated and purified according to the literature, with minor modifications.[49] The bacterial pellet was suspended and incubated with 15 mg/ml lysozyme (Sigma-Aldrich Co., LCC) at

37°C for 1 h in TE10. Purified achromopeptidase (Wako Pure Chemical Industries, Ltd) was added at a final concentration of 2000 units/ml and then incubated at 37°C for 30 min. The suspension was treated with 1% (wt/vol) sodium dodecyl sulphate and 1 mg/ml proteinase K (Merck Japan) and incubated at 55°C for 1 h. The lysate was treated with phenol/chloroform/isoamyl alcohol (Life Technologies Japan, Ltd). DNA was precipitated by adding ethanol and pelleted by centrifugation at 3,300 *g* at 4°C for 15 min. The DNA pellet was rinsed with 75% ethanol, dried, and dissolved in 10 mM Tris-HCl/1 mM EDTA (TE). DNA samples were purified by treating with 1 mg/ml RNase A (Wako Pure Chemical Industries, Ltd) at 37°C for 30 min and precipitated by adding equal volumes of 20% polyethylene glycol solution (PEG6000-2.5M NaCl). DNA was pelleted by centrifugation at 8,060 *g* at 4°C, rinsed with 75% ethanol, and dissolved in TE.

2.4. *454 barcoded pyrosequencing of 16S rRNA gene*

The V1–V2 region of the 16S rRNA gene was amplified using forward primer (5′-CCATCTCATCCCTGCG TGTCTCCGACTCAGNNNNNNNNNNagrgtttgatymtggc tcag-3′) containing the 454 primer A, a unique 10-bp barcode sequence for each sample (indicated in N), and 27Fmod (5′-agrgtttgatymtggctcag) in which the third base A in the original primer 27F was changed to R, and reverse primer (5′-CCTATCCCCTGTGTGC CTTGGCAGTCTCAGtgctgcctcccgtaggagt-3′) containing the 454 primer B and reverse primer 338R (5′-tgctgcctcccgtaggagt). PCR was performed in 1 × Ex Taq PCR buffer (50 μl), deoxynucleoside triphosphate (2.5 mM), Ex Taq polymerase (Takara Bio, Inc., Shiga), each primer (10 μM), and 40 ng of extracted DNA under conditions of 2 min at 96°C, 20 cycles of 96°C for 30 s, 55°C for 45 s, and 72°C for 1 min, and a final extension of 72°C for 10 min on a 9700 PCR system (Life Technologies Japan, Ltd, Tokyo, Japan). PCR products of approximately 370 bp were confirmed by agarose gel electrophoresis, purified by AMPure XP magnetic purification beads (Beckman Coulter, Inc., Brea, CA, USA), and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies Japan, Ltd, Tokyo, Japan). Mixed samples were prepared by pooling approximately equal amounts of PCR amplicons from each sample and subjected to 454 GS FLX Titanium or 454 GS JUNIOR (Roche Applied Science) sequencing according to the manufacturer's instructions.

2.5. *Analysis pipeline for 454 barcoded pyrosequencing of 16S PCR amplicons*

We developed an analysis pipeline for 454 barcoded pyrosequencing of PCR amplicons of the V1-2 region amplified by 27Fmod-338R primers. First, 16S reads were assigned to each sample based on the barcode sequence information. Second, 16S reads that did not have PCR primer sequences at both sequence termini and those with an average quality value < 25 were filtered out. Third, 16S reads containing possible chimaeric sequences that had BLAST match lengths of $< 90\%$ with reference sequences in the database were removed. Reads removed in these processes accounted for about 35% of all reads, most of which represented reads lacking PCR primer sequences (Supplementary Table S2). Finally, filter-passed reads were obtained for further analysis by trimming off both primer sequences.

All 3000 filter-passed reads of the 16S V1-2 sequences obtained from each subject were deposited in DDBJ/GenBank/EMBL with accession numbers DRA000869–DRA000886.

2.6. *Assessment of the quantitative accuracy of 16S data using artificial bacterial communities*

Two artificial bacterial communities (designated 'mock01' and 'mock02') were constructed by mixing genomic DNA from 10 and 11 different human gut-associated bacterial strains with an appropriate ratio, respectively (Supplementary Table S3). Genome sequences of these microbes were completely sequenced and are publicly available. From these communities, we amplified the V1-2 region by PCR using 27F-338R and 27Fmod-338R primers, the V5-6 region by 787F-1061R primers, and the V1-9 region by 27F-1492R primers. V1-2 and V5-6 amplicons were subjected to 454 pyrosequencing, and V1-9 amplicons were cloned in *Escherichia coli*, and 3000 clones were sequenced by the Sanger method, and the products were analysed with the ABI3730xl (Life Technologies Japan, Ltd, Tokyo). We also performed duplicate qPCR experiments targeting a specific genomic region of the bacterial strains in the two mock communities. All filter-passed 16S *de novo* sequences and qPCR data were then analysed by principal component analysis (PCA) to compare and assess the quantitative accuracy (Fig. 1).

The error rate of the filter-passed sequences using 27Fmod-338R primers obtained from the two mock communities was estimated by aligning the 16S V1-2 *de novo* sequences with the reference 16S sequences in the two mock communities (Supplementary Table S4).

2.7. *Data analysis*

2.7.1. Database Two databases were constructed for the analysis of 16S sequences. One is the 16S rRNA gene sequence database constructed

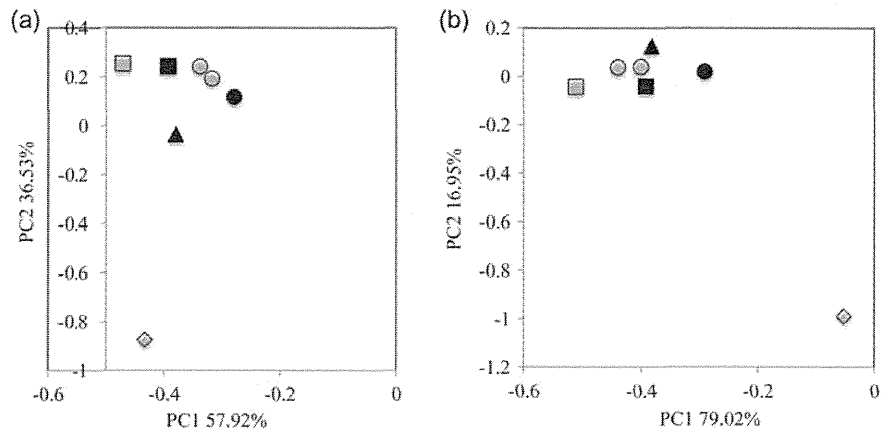

Figure 1. Assessment of the quantitative accuracy of the analysis of the bacterial composition of two mock communities by various methods. PCA analysis of the data was obtained from various methods using mock01 (a) and mock02 (b). Closed circle: expected, open circle: duplicate qPCR, closed square: pyrosequencing of 16S V1-2 region using 27Fmod, open square: pyrosequencing of 16S V1-2 region using 27F, closed triangle: pyrosequencing of 16S V5-6 region, open diamond: Sanger sequencing of nearly full-length 16S clone.

by collecting 16S sequences of ≥1200 bp of bacterial isolates from the Ribosomal Database Project v. 10.27. Another database is the reference genome database constructed by collecting genome sequences from the NCBI FTP site (ftp://ftp.ncbi.nih.gov/genbank/, Dec 2011) that includes 1482 complete and 605 draft bacterial genomes.

2.7.2. Operational taxonomic unit (OTU) and UniFrac distance analysis We used 3000 filter-passed reads of 16S sequences for operational taxonomic unit (OTU) and UniFrac distance analysis for each sample. For OTU analysis, clustering of 16S reads was done using a 96% pairwise-identity cutoff with the UCLUST program (www.drive5.com). Representative sequences for each OTU were assigned to bacterial species by BLAST search with a 96% pairwise-identity cutoff against the two databases mentioned above. UniFrac distance analysis was used to determine the dissimilarity (distance) between two communities based on the fraction of branch length shared between two communities within a phylogenetic tree constructed from 16S sequence datasets.[44]

2.7.3. Other Estimation of OTU numbers by extrapolation (Chao1 and ACE) was calculated with the vegan package (v2.0-5) for R (v2.15.2).

3. Results and discussion

3.1. Quantitative accuracy of 16S data produced by 454 pyrosequencing

Pyrosequencing of PCR amplicons of bacterial 16S short variable regions is the most popular and a high-throughput approach to infer and characterize the species composition in bacterial communities.[42,45,46,48] The 454 pyrosequencing platform, which can produce over 400 bases per read, is also superior to shorter read-length sequencers with respect to sequence accuracy for single-end sequencing.[50,51] However, this PCR-based method has a problem particularly in quantification of the composition of the genus *Bifidobacterium*, a dominant species in human gut microbiota because the 16S sequence of *Bifidobacterium* has a few base mismatches with the commonly used PCR primer 27F (or 8F), underestimating this genus in the community.[52–55] To improve this, we modified primer 27F to 27Fmod by changing the third base G to R (G or A) in 27F-YM[53] that perfectly matched with the annealing site of the *Bifidobacterium* 16S gene (see Materials and methods).

To assess the 16S data using 27Fmod, we compared various 16S sequence and qPCR data obtained from two mock communities (Supplementary Table S3) that are useful to evaluate the quantitative accuracy of 16S-based data and the sequencing error rate.[56,57] Quantitative accuracy of the overall bacterial composition was evaluated by comparing the similarity of each data to the expected ('Expected') using PCA (Fig. 1). From the PCA data, Euclidean distance was calculated for evaluation of the similarity of each data with the 'Expected'. The results revealed that the order of their similarities with the 'Expected' was the qPCR data ≥ the V1-2 data using 27Fmod > the V5-6 data > the V1-2 data using 27F >> the data of Sanger sequencing-based full-length V1-9, indicating that the use of 27Fmod greatly improved the quantitative accuracy for evaluation of the overall

bacterial composition (Supplementary Table S5). This improvement was largely dependent on the improved estimation of the *Bifidobacterium* content by the use of 27Fmod. The average relative *Bifidobacterium* content in the two mock communities estimated from the data of V1-2 using 27F was only 1.5% of the 'Expected' (100%), whereas the use of 27Fmod increased the relative content to 61% that was also better than that estimated from the data of V5-6 and Sanger full-length analyses (Supplementary Fig. S2). Because qPCR can be used only when genomes of all bacteria in a given community are known, or only for a limited number of specific known species, we concluded that 454 pyrosequencing of the V1-2 region using 27Fmod-338R provided more quantitatively accurate data for bacterial composition in human gut microbiota than that using the conventional 27F primer.

We estimated the average error rate of filter-passed V1-2 data using 27Fmod-338R by aligning the V1-2 and reference 16S sequences of bacterial strains used in the two mock communities. The error rate was estimated to be 0.58 and 0.66% for mock01 and mock02 by local alignment, respectively (Supplementary Table S4). These error rates are similar to the previously published data,[43,45,50] but lower than in another study.[58] The latter may be due to differences in the examined alignment length and between local and global alignments. Errors in 454 pyrosequencing data can be the primary cause for overestimation of the OTU number that is an issue which needs to be improved for accurate estimation of species richness in bacterial communities.[59,60] We compared OTU numbers generated from clustering of various qualities of 16S reads with a 96% and a 97% pair-wise identity cutoff. For this comparison, we made and used three datasets: only primer check-passed reads having the highest error rates, filter-passed reads, and selected filter-passed reads having the lowest error rates. The results indicated that a 96% cutoff clustering of error-rich reads and a 97% cutoff clustering of filter-passed reads gave the worse results than a 96% cutoff clustering of filter-passed and selected filter-passed reads (Supplementary Fig. S3). A 97% cutoff was defined for clustering of highly accurate Sanger full-length 16S sequences.[61] Therefore, in clustering of pyrosequencing data having higher error rate than Sanger data, the use of a cutoff identity lower than 97% and a lower number of reads are reasonable to reduce overestimation of the OTU number. A 96% cutoff clustering of filter-passed reads gave similar OTU numbers up to 30–50 reads to those of filter-passed reads having the lowest error rates. These read numbers are approximately three to five times the number of input strains. After several trials testing the mock communities, we decided to use 3000–5000 reads per sample for clustering with a 96% cutoff for the analysis of human gut microbiota. Indeed, OTU numbers using a 96% cutoff clustering of 3000 reads decreased about 15% when compared with those using a 97% cutoff clustering.

3.2. *Species richness and diversity in human faecal microbiota with probiotic intervention*

We randomly selected 3000 reads of 16S V1-2 sequences from all filter-passed reads for each sample (Supplementary Table S2) and used 474 000 reads in total from 158 faecal DNA samples of 18 subjects for the analysis of species richness and composition in human gut microbiota. Clustering of all reads with a 96% pairwise-identity cutoff gave a total of 2758 OTUs. After removing the minority OTUs having $<0.1\%$ abundance in any samples, 1175 OTUs having $\geq 0.1\%$ abundance in at least 1 sample, accounting for 99.1% of all 16S reads, were used for further analysis.

3.2.1. Detection of administrated probiotic strains in faecal samples We investigated whether administrated strains contained in the probiotic products can be detected in faecal DNA. We sequenced the 16S V1-2 region of all bacterial strains contained in probiotic products used in this study. The BLAST search to the databases indicated that except for the *Bifidobacterium longum* strain used in Group III, the 16S sequences of all strains in the probiotic products significantly differed from those of the indigenous species phylogenetically closest to the probiotic strains. The 16S sequence of the *B. longum* strain used in Group III was almost identical to that of an indigenous *Bifidobacterium* species, so that we used a distinguishable additive *Lactococcus lactis* strain in this product for the detection of administrated bacteria in Group III samples. The 16S sequences of these probiotic strains were included in the databases constructed in this study, and the 16S reads assigned to administrated strains had the average similarity between 99.4 and 99.9% identities with the reference sequences (data not shown). The 16S reads assigned to probiotic or additive strains were detected in samples (S01–S04) during probiotic intervention [designated 'Pro(+)'] at various frequencies, but almost none were detected in samples (S00 and S05–S08) without probiotic administration [designated 'Pro(−)'] (Supplementary Table S6). The administrated probiotic strains were shown to be more frequently detected in samples during the intervention than in the pre- and post-intervention periods using different detection methods such as culturing, targeted PCR, and hybridization.[24,26–28,30,32,33,62] In

the present study, two probiotic *Lactobacillus* and one additive *Lactococcus* strains were detected in post-intervention samples in three subjects with a minimum count, respectively. The similarity of three 16S sequences was 99.4, 99.7, and 100% identity with those of administrated *Lactobacillus* and *Lactococcus* strains, indicating that these are administrated strains. The survival of some probiotic strains in the post-intervention period was also reported previously.[28,30] Our data suggested that some probiotic strains seem to be able to persistently colonize the intestine and their survivability may be related to metabolic activity in the intestine.[63,64] Probiotic *Bifidobacterium* strains were not detected in any Pro(−) samples. However, we found two distinct 16S sequences both assigned to *Bifidobacterium animalis* in two subjects APr37 and APr39. One showed a high similarity of >98% identity with the 16S sequence of the administrated *B. animalis* and was detected with high frequency only in the Pro(+) samples, whereas another showed a low similarity of 96.5–97.4% identity (a mean of 97.2%) with low frequency in both the Pro(−) and Pro(+) samples. These data suggest the presence of unknown indigenous species phylogenetically close to, but distinct from, probiotic *B. animalis* in human gut microbiota. The total number of bacteria contained in each probiotic product was varied between 10^9 and 10^{10}, showing no large difference in quantity among them (Supplementary Table S1). No clear correlation was also observed between the number of bacteria in the products and the frequency in detection of the administrated strains in the Pro(+) samples. From these observations, the frequency of administrated bacteria detected in faeces may not be largely affected by their amounts in the products. Therefore, detection of *Lactobacillus brevis* and *Lactobacillus delbrueck* at relatively low level in faeces cannot be simply explained by the difference in a dose, but could be considered the association with several factors such as their survivability in the intestine, diet, or physiological conditions of subjects.

3.2.2. Change of species richness in samples with and without probiotics We analysed species richness (OTU number) in the Pro(+) and Pro(−) samples. Supplementary Figure S4 shows the change in OTU numbers for every sample in each subject, indicating that OTU numbers vary dramatically for every sample. Most of the variation can be attributed to single OTUs representing the minority species. We averaged the OTU numbers of the Pro(−) and Pro(+) samples and compared them for subject, group, type of probotics (*Lactobacillus* and *Bifidobacterium*), and all combined samples, respectively (Fig. 2). The average OTU numbers in 6 out of 18 subjects were decreased in the range of the ratio of 0.83–0.95 in the Pro(+) samples when compared with the Pro(−) samples, whereas those in other 12 subjects were increased in the range of the ratio of

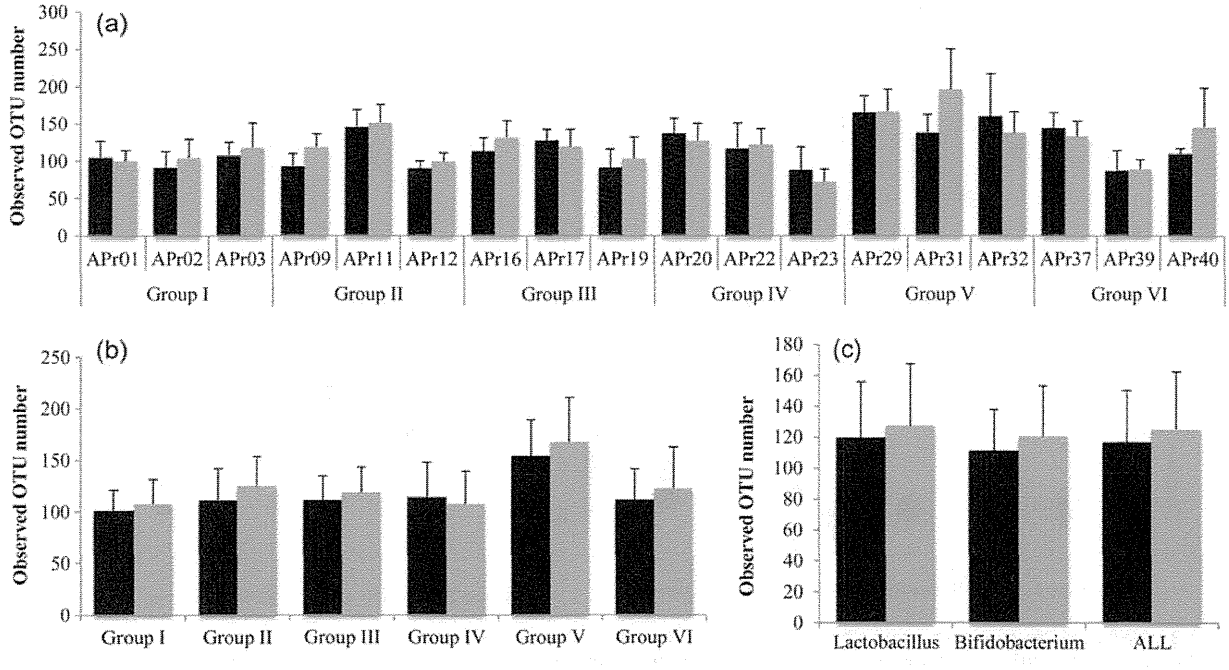

Figure 2. Change in OTU number in faecal microbiota with and without probiotic administration. (a) Individual, (b) group, (c) type of probiotics. Black bar indicates Pro(−) samples. Grey bar indicates Pro(+) samples. The error bars represent standard deviation.

1.01−1.43. For group, only Group IV showed a decrease in the average OTU number in the Pro(+) samples with the ratio of 0.94. For type of probiotics and all samples, the average OTU numbers in the Pro(+) samples were slightly more abundant (approximately 1.07-fold) than those in the Pro(−) samples, but no statistical significance was observed in any dataset. The increase in OTU number in the Pro(+) samples was largely due to the minority species (Supplementary Fig. S4), whereas the abundance of the majority species (OTUs containing ≥10 reads) was almost constant over time. We performed the same analysis using different sets of 3000 reads for each subject. The analysis reproducibly showed the similar pattern and the degree of the change in OTU numbers to which the minority species is largely attributed (data not shown). These data indicate that administration of probiotics tends to increase species richness in faecal microbiota that may be beneficial for the consumer because the species richness in faecal microbiota of subjects afflicted with disease such as inflammatory bowel disease is significantly reduced when compared with that of healthy subjects.[65]

3.2.3. Change of species composition in samples with and without probiotics We obtained the average weighted and unweighted UniFrac distances within Pro(−), within Pro(+), and between Pro(−) and Pro(+) samples for every group, probiotic types, and all subjects, respectively (Fig. 3). High UniFrac distance implies high variability of microbiota structure within and between samples. If the difference between any pair of the three distances is statistically significant, it can be considered that probiotic administration significantly affected the overall microbiota composition. We found the largest difference between weighted UniFac distances of the Pro(+)

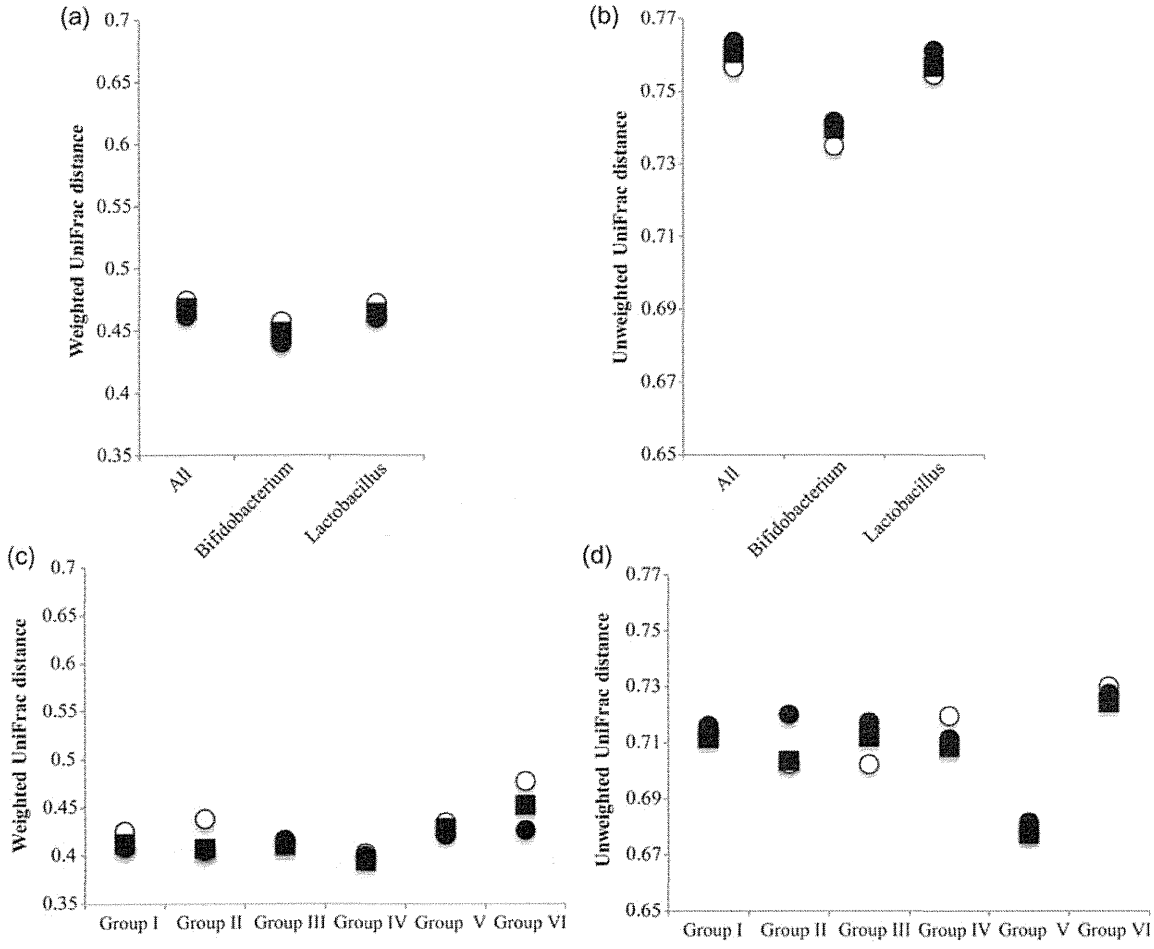

Figure 3. Average UniFrac distance within Pro (−) and Pro(+) and between Pro(−) and Pro(+) for each group, type of probitotics, and all subjects. Average UniFrac distance between any pair of the three distances for type of probiotics and all subjects (a and b), and each group (c and d). Open circle, closed circle, and closed square indicate average UniFrac distance within Pro (−), within Pro (+), and between Pro(−) and Pro(+) samples, respectively.

and Pro(−) samples in Group VI. However, statistical evaluation of this difference by the Student's *t*-test showed no significance (*P*-value > 0.05) for 781 out of 1000 times (Supplementary Table S7). These data imply high stability of gut microbiota to probiotic administration for all subjects examined. We also analysed UniFrac distances of intra-subject gut microbiota (Fig. 4). Although 5 subjects (APr02, 12, 16, 37, and 39) showed a significant difference in the UniFrac distances between Pro(−) and Pro(+) samples, the results showed that both weighted and unweighted distances between Pro(−) and Pro(+) of all intra-subjects were significantly lower than the average distance of the 18 unrelated subjects. The Welch's *t*-test for these differences showed statistical significance (Supplementary Table S8). We also performed the UniFrac distance analysis using different 16S datasets of 5000 reads for group, type of probiotics, all subjects, and intra-subject. The results similarly showed no statistical significance in differences between any pair of the 3 UniFrac distances and the significantly lower UniFrac distance of each intra-subject than that of the 18 unrelated subjects (data not shown). Thus, these data suggested that the perturbation of microbiota elicited by probiotics in an intra-subject did not overcome the inter-subject variations of gut microbiota, supporting high intra-specificity and stability of gut microbiota.[66,67] This robustness of gut microbiota of adults is in contrast with the profound effect of antibiotic administration on adult gut microbiota[68] and the observed response of gut microbiota of infants fed with probiotics, in which the infant gut microbiota composition was considerably affected by probiotics.[36] A short-term dietary intervention study showed that in controlled feeding of the same diet to subjects over 10 days, a marked change was observed within 1 day after the intervention initiation.[69] In the present study, no significant difference was observed between samples before (S00) and first samples (S01) after the intervention initiation (data not shown). It would be valuable to analyse faecal samples collected within a few days after administration of probiotics for evaluation of the short-term effect of probiotics.

4. Identification of bacterial species showing significant increase or decrease by probiotic administration

Although our results suggested that administration of probiotics had almost no effect on the overall structure of gut microbiota, it is possible to identify bacterial species largely responding to the administrated probiotics at the OTU/species level. We surveyed OTUs showing an increase or a decrease between the Pro(+) and Pro(−) samples by comparing the number of 16S reads for each OTU. We first enumerated the OTUs showing ≥2-fold change between the

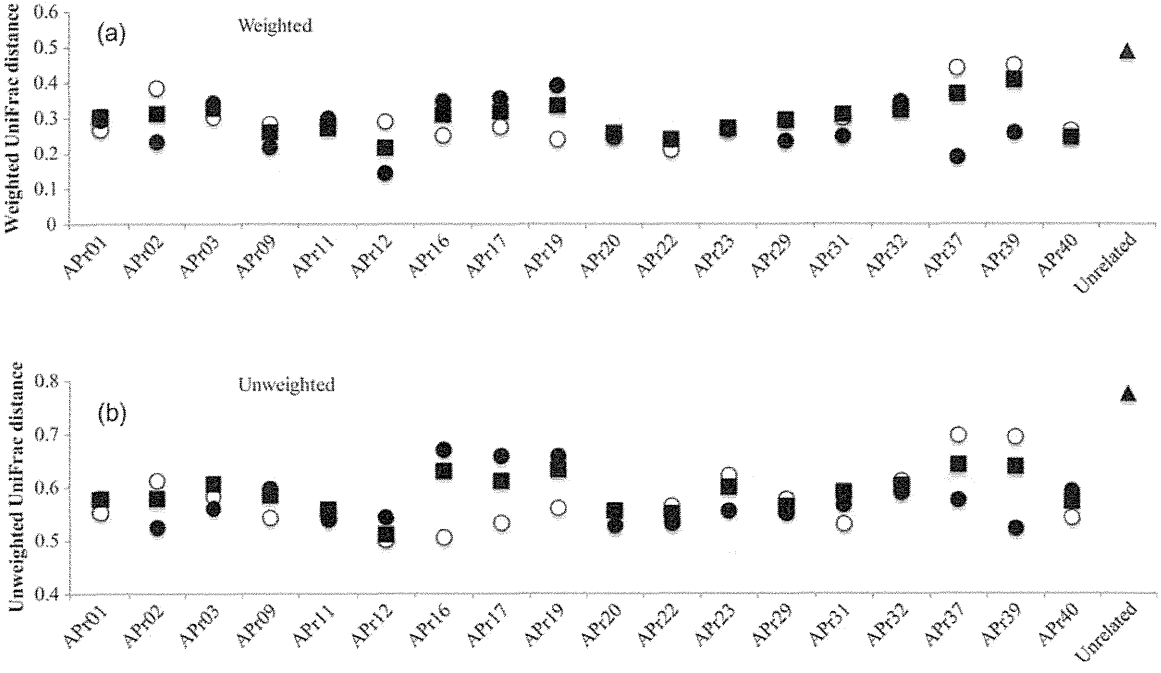

Figure 4. Average UniFrac distance within Pro(−) and Pro(+) and between Pro(−) and Pro(+) for each subject. Open circles, closed circles, and closed squares indicate average UniFrac distance within Pro(−), within Pro(+), and between Pro(−) and Pro(+) samples, respectively. Closed triangles indicate average UniFrac distance between samples (S00) of 18 unrelated individuals.

Pro(−) and Pro(+) samples for each subject, and the quantity difference was also obtained by subtracting the 16S read number of the Pro(+) samples from that of the Pro(−) samples. This is because OTUs showing a high quantity difference, but less fold change may also have substantial influence on gut microbiota composition. We found several OTUs significantly changed by probiotic administration, including OTUs assigned to both the indigenous and administrated strains (Fig. 5). We listed 88 OTUs (7.5% of all analysed 1175 OTUs) showing significant change of ≥3-fold, among which 30 OTUs changed by ≥10-fold (Supplementary Fig. S5). We excluded 6 OTUs assigned to the administrated strains from the 30 OTUs and obtained 24 OTUs assigned to the indigenous species, including OTU00072 assigned to *Streptococcus salivarius* that showed significant change in 2 subjects (Supplementary Table S9). We also found seven OTUs showing significant difference in quantity between both samples (Supplementary Table S10). Of the combined 32 OTUs (2.7%), 18 were increased and 14 were decreased by probiotic administration. Many of the OTUs showing a significant increase were assigned to minority species in the Pro(−) samples, but some increased up to nearly 7% in abundance (e.g. OTU00372 assigned to *Eubacterium rectale*). On the other hand, the OTUs showing a significant decrease were almost undetected in the Pro(+) samples. Phylum-level species assignment showed that species belonging to the phylum *Firmicutes* were most largely affected by both probiotics, and all species belonging to the phylum *Bacteroidetes* were affected only by *Lactobacillus* probiotics (Table 1). The 32 OTUs were assigned to 27 indigenous species, among which 4 species (*Clostridium clostridioforme*, *Eubacterium eligens*, *E. rectale*, and *Faecalibacterium prausnitzii*) were assigned by 8 different OTUs and 1 species (*S. salivarius*) was assigned by the 2 same OTUs as described above. All these species except for *S. salivarius* were found to show significant change only in one subject, indicating that response of the indigenous species to probiotics is highly individual specific (Supplementary Fig. S6). Two different OTUs (OTU02677 and OTU02748) assigned to *F. prausnitzii*, of which the reduction is known to be correlated with inflammatory bowel disease,[70] were found to both decrease and increase in the same subject (APr40) by probiotic administration, suggesting that these two phylogenetically close species may have the diversity of response to probiotic action. We also examined distribution of the 32 OTUs in the subjects. The results revealed that 4 subjects

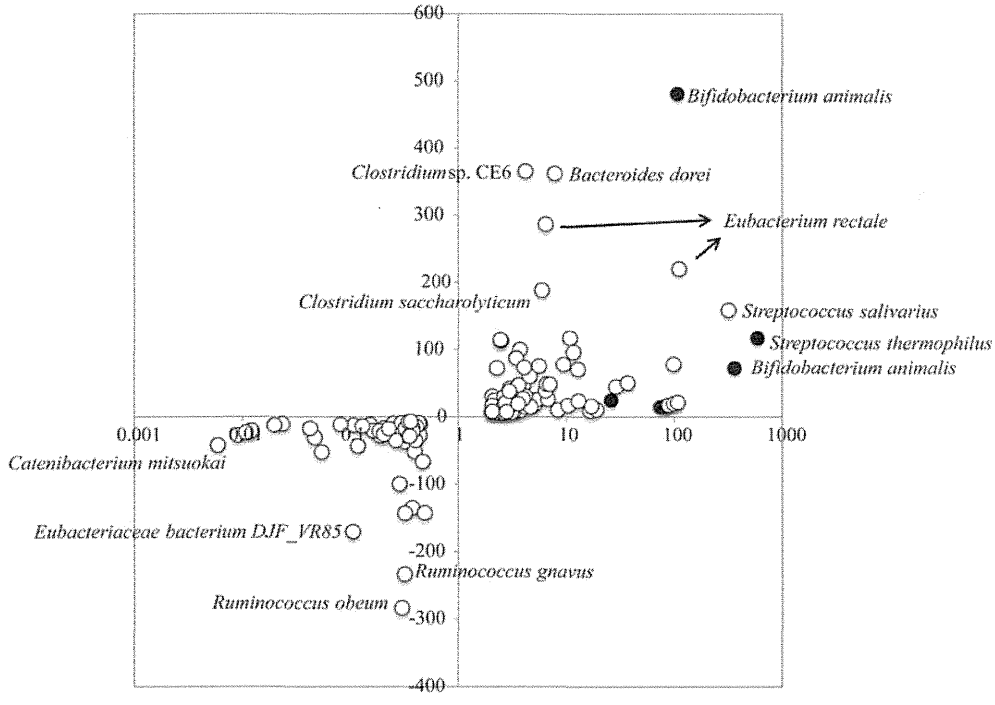

Figure 5. OTUs showing ≥2-fold change and their difference in quantity between the Pro(−) and Pro(+) samples. The *x*-axis represents the scale of fold change between the Pro(+) and Pro(−) samples. The *y*-axis represents the difference (number of reads) in quantity between the Pro(+) and Pro(−) samples. Closed and open circles indicate the administrated probiotic and indigenous species, respectively.

Table 1. Phylum-level species assignment of OTUs showing significant fold change or quantity difference by administration of probiotics

Type of probiotics	Change	[a]Number of varied OTUs	Fold change (≥10-fold)				Number of varied OTUs	Difference (≥150 reads)	
			Firmicutes	Actinobacteria	Bacteroidetes	Unclassified bacterium		Firmicutes	Bacteroidetes
Lactobacillus	Increase	9	7	0	1	1	3	2	1
	Decrease	7	3	1	3	0	1	1	0
	Total	16	10	1	4	1	4	3	1
Bifidobacterium	Increase	5	5	0	0	0	1	1	0
	Decrease	4	4	0	0	0	2	2	0
	Total	9	9	0	0	0	3	3	0
All	Increase	14	12	0	1	1	4	3	1
	Decrease	11	7	1	3	0	3	3	0
	Total	25	19	1	4	1	7	6	1

[a]Administrated probiotic strains were excluded, and only OTUs with a P-value < 0.05 are shown.

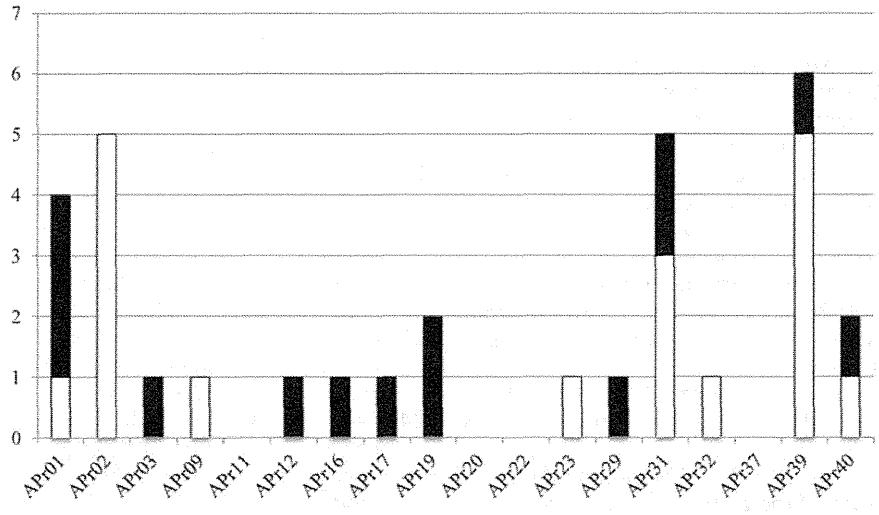

Figure 6. Distribution of 32 OTUs showing a significant change in 18 subjects. The y-axis indicates the number of OTUs showing significant change between the Pro(−) and Pro(+) samples in each subject (see Supplementary Tables S9 and S10). Open and closed bars indicate increased and decreased OTUs, respectively.

(APr11, 20, 22, and 37) did not have such OTUs and 8 subjects had only 1 OTU, whereas 4 subjects (APr01, 02, 31, and 39) had more than 4 OTUs showing significant change (Fig. 6), suggesting their uneven distribution in the 18 subjects. These data imply existence of the sensitive and less sensitive responders to probiotic action and if so, it would be interesting to investigate the relation between gut microbiota type and its response to probiotics.

In summary, we analysed changes of the gut microbiota composition of healthy adults fed with probiotics using the 454 pyrosequencing platform with the improved quantitative accuracy for evaluation of the overall bacterial composition. The present study using large datasets enabled us to more comprehensively and precisely evaluate the effect of probiotics on gut microbiota than the previous probiotic intervention researches in which the analysis exclusively focussed on only several limited bacterial species using conventional methods. Our data further support the high inter-subject variability and the high intra-subject stability that is the current common view for the feature of adult gut microbiota. A recent study of gut microbiota in twins demonstrated that probiotics had almost no effect on the community structure, but affected the gene expression of microbiota.[39] To more deeply understand the potential function of probiotics, the analysis of bacterial and host cell's transcriptome and intestinal metabolome is required.

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LETTER

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T_{reg} induction by a rationally selected mixture of Clostridia strains from the human microbiota

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Manipulation of the gut microbiota holds great promise for the treatment of inflammatory and allergic diseases[1,2]. Although numerous probiotic microorganisms have been identified[3], there remains a compelling need to discover organisms that elicit more robust therapeutic responses, are compatible with the host, and can affect a specific arm of the host immune system in a well-controlled, physiological manner. Here we use a rational approach to isolate $CD4^+FOXP3^+$ regulatory T (T_{reg})-cell-inducing bacterial strains from the human indigenous microbiota. Starting with a healthy human faecal sample, a sequence of selection steps was applied to obtain mice colonized with human microbiota enriched in T_{reg}-cell-inducing species. From these mice, we isolated and selected 17 strains of bacteria on the basis of their high potency in enhancing T_{reg} cell abundance and inducing important anti-inflammatory molecules—including interleukin-10 (IL-10) and inducible T-cell co-stimulator (ICOS)—in T_{reg} cells upon inoculation into germ-free mice. Genome sequencing revealed that the 17 strains fall within clusters IV, XIVa and XVIII of Clostridia, which lack prominent toxins and virulence factors. The 17 strains act as a community to provide bacterial antigens and a TGF-β-rich environment to help expansion and differentiation of T_{reg} cells. Oral administration of the combination of 17 strains to adult mice attenuated disease in models of colitis and allergic diarrhoea. Use of the isolated strains may allow for tailored therapeutic manipulation of human immune disorders.

$CD4^+FOXP3^+$ T_{reg} cells are present most abundantly in the intestinal mucosa at steady state, and contribute to intestinal and systemic immune homeostasis[4–7]. In germ-free mice, the frequency of colonic T_{reg} cells and levels of IL-10 expression by T_{reg} cells are markedly reduced[4–7]. We have shown previously that a combination of Clostridia strains isolated from conventionally reared mice potently affect the number and function of $CD4^+FOXP3^+$ T_{reg} cells in mouse colonic lamina propria[4]. In an attempt to enable clinical translation of our previous findings, we aimed to identify T_{reg}-cell-inducing bacterial strains derived from the human microbiota (see Supplementary Fig. 1 for a summary of the procedure).

We obtained a human stool sample from a healthy Japanese volunteer. Because we previously reported that the chloroform-resistant fraction of mouse gut microbiota was enriched in T_{reg}-cell-inducing species[4], the stool sample was either untreated or treated with chloroform and orally inoculated into IQI/Jic germ-free mice. Each group of ex-germ-free (exGF) mice was separately housed for 3–4 weeks in vinyl isolators to avoid further microbial contamination. Although a recent study showed that the human microbiota had no impact on the immune responses in the mouse small intestine[8], we observed a significant increase in the percentage of $FOXP3^+$ T_{reg} cells among $CD4^+$ T cells in the colons of exGF mice inoculated with untreated human faeces compared with germ-free mice (Fig. 1a and Supplementary Fig. 2). Notably, a more pronounced increase was observed in the colons of exGF mice inoculated with chloroform-treated human faeces (Fig. 1a). These findings suggest that the human intestinal microbiota contains T_{reg}-cell-inducing bacteria, and that they are enriched in the chloroform-resistant fraction. We also examined the effects of human faeces inoculation on colonic IL-17- and IFN-γ-expressing $CD4^+$ cells (T_H17 and T_H1 cells). In exGF mice inoculated with untreated or chloroform-treated human faeces, the frequency of T_H1 cells was unchanged compared with germ-free mice (Fig. 1b). By contrast, there

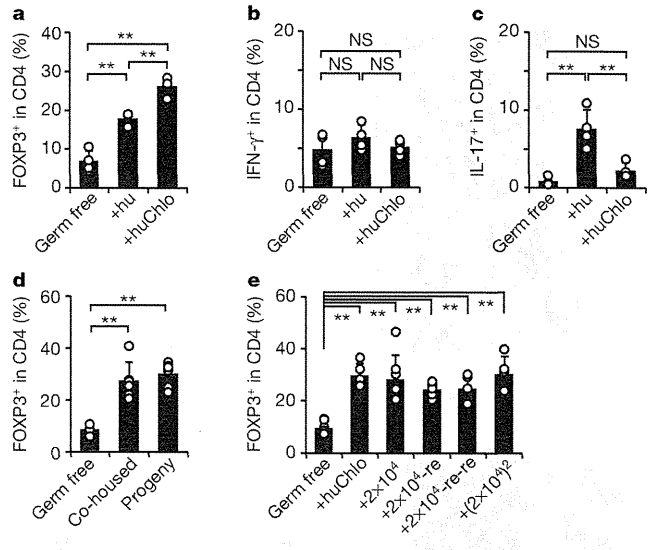

Figure 1 | T_{reg} cell accumulation in germ-free mice induced by inoculation with human microbiota. a–e, The percentages of $FOXP3^+$, $IL\text{-}17^+$ and $IFN\text{-}\gamma^+$ cells within the $CD4^+$ cell population in the colon lamina propria of the indicated mice are shown (see also Supplementary Fig. 2). Circles represent individual animals. The height of the black bars indicates the mean. All experiments were performed more than twice with similar results. Error bars indicate s.d. $^{**}P < 0.01$; NS, not significant. +hu, exGF mice inoculated with untreated human faeces; +huChlo, exGF mice inoculated with chloroform-treated human faeces. (See the main text for further definitions of *x*-axis labels.)

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