

Changes in the gut microbiota composition and the plasma ghrelin level in patients with *Helicobacter pylori*-infected patients with eradication therapy

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ABSTRACT

Objective To investigate the influence of antimicrobials on both the gut microbiota structure and the plasma ghrelin level using *Helicobacter pylori*-infected patients who underwent eradication therapy.

Design Twenty *H. pylori*-infected patients (mean age 68.3 years old) who underwent eradication therapy participated in the study. For the therapy, patients had 1 week of triple therapy consisting of amoxicillin, clarithromycin and proton-pump inhibitors. Stool and blood samples were obtained before (S1), immediately after (S2) and/or 3 months after (S3) the therapies. The concentrations of ghrelin and leptin in the blood were assayed using an ELISA. The V3-V4 region of the 16S rRNA gene was amplified using bacterial DNA from the stool, and about 50 000 high-quality amplicons per sample were grouped into operational taxonomic units for bacteriological analyses.

Results The Bacteroidetes:Firmicutes (B:F) ratio was significantly greater at S3 than S1 ($P<0.01$). This increase in the B:F ratio between S3 and S1 was found in 15 out of 20 patients. A significant decrease in the concentration of active ghrelin ($P=0.003$) in the plasma was observed between S3 and S1. There was a statistically significant correlation between the rate of patients whose B:F ratio increased and that of patients whose active ghrelin level decreased between S3 and S1 according to Fisher's exact probability test ($P=0.03$).

Conclusions Changes in the gut microbiota, such as the B:F ratio after treatment with antimicrobials, might cause a change in the plasma ghrelin level, as the direct and earliest target of antimicrobials would be the microbiota rather than the hormone-secreting system.

INTRODUCTION

Although antimicrobials have definite effects on particular pathogenic bacteria, the effect of antimicrobials on the gut microbial community and metabolism has recently been explored. Many of those studies performed so far are characterised by significant heterogeneity with respect to type of antimicrobials

Summary box

What is already known about this subject?

- ▶ Gut microbiota plays a fundamental role in the pathophysiology of obesity.
- ▶ Ghrelin is associated with obesity after the eradication of *Helicobacter pylori*.

What are the new findings?

- ▶ Alteration in the gut microbiota may affect the plasma ghrelin level.

How might it impact on clinical practice in the foreseeable future?

- ▶ Both the gut microbiota profiling and plasma ghrelin level might be used as an earliest biomarker to foresee the insulin resistance or obesity.

tested, route of administration, duration of follow-up and so on. However, in the eradication therapy of *Helicobacter pylori*, the first-line therapy has been widely used, which consists of oral administration of clarithromycin, amoxicillin and a proton-pump inhibitor twice a day for 7 days.¹ Therefore, it will be free from the problem of heterogeneity to use *H. pylori*-infected patients with eradication therapy as subjects in the study of the effect of antimicrobials on indigenous bacteria in human.

It was reported that the body weight and body mass index significantly increased 1 year after the eradication of *H. pylori* when examined in 579 Japanese patients.² Ghrelin is a multifunctional hormone that facilitates fat storage and regulates body weight.³ As ghrelin is predominantly synthesised and secreted by the gastric mucosa, the obesity associated with eradication of *H. pylori* has been attributed to an increase in the

ghrelin level.⁴ However, some groups^{5,6} have found that the plasma ghrelin concentration decreased in most patients 12 weeks after successful *H. pylori* eradication therapy. A recent systemic review also reported that the relationship between circulating ghrelin and eradication of *H. pylori* is more complex.⁷

Recent studies have also suggested that the gut microbiota play a fundamental role in the pathophysiology of obesity.⁸ Ley *et al.*⁹ reported that the ratio of the phylum Bacteroidetes to the phylum Firmicutes (B:F ratio) in the faeces increased when weight was reduced either by a fat-restricted or carbohydrate-restricted diet in humans, suggesting a critical role of the gut microbiota in the obesity.

Therefore, in the present study, we investigated the influence of antimicrobials on both the gut microbiota structure and the plasma ghrelin level, as we hypothesised that the change in the ghrelin level after *H. pylori* eradication therapy was caused by elimination of *H. pylori* in the stomach but also by an alteration in the gut microbiota due to antimicrobials.

METHODS

Patients

Patients were defined as positive for *H. pylori* infection if positive results were obtained on a ¹³C-urea breath

test, *H. pylori* stool antigen test or culturing of biopsy specimens. The exclusion criteria were the patients with gastric cancers and a history of gastrointestinal or hepatobiliary surgery. The demographic data and clinical characteristics of the subjects are summarised in table 1. For eradication therapy, patients had 1 week of triple therapy consisting of lansoprazole (30 mg twice a day), amoxicillin (750 mg twice daily) and clarithromycin (200 mg twice daily). The eradication of *H. pylori* was confirmed by a urea breath test 3 months after the termination of eradication therapy. Written informed consent was obtained from all of the patients.

Hormone assay

The concentrations of des-acyl ghrelin and active ghrelin in the plasma were assayed using a Desacyl-Ghrelin ELISA Kit (SCETI, Tokyo, Japan) and Active Ghrelin ELISA Kit (SCETI), respectively. The specific antibodies used in the former and latter assay kits recognise the C-terminal portion and the octanoyl-modified Ser 3 residue of ghrelin, respectively.¹⁰ The concentration of leptin in the serum was assayed using Human Leptin RIA Kit (Millipore, Belmopan, Belize).

Stool sample collection and DNA extraction

Stool sample collection from patients was done three times before (S1), immediately after (S2) and 3 months

Table 1 Demographic and clinical data of the patients

ID of the patient	Age	Gender	Endoscopy of the stomach	After eradication therapy		
				Eradication	B:F ratio	Ghrelin level
1	47	F	AG	Success	Down	Up
2	66	F	AG	Success	Down	Up
3	42	M	AG	Success	Up	Down
4	62	F	AG	Success	Up	Down
5	73	F	AG	Success	Down	Up
6	42	M	AG, RE	Success	Down	Down
7	80	M	AG	Success	Up	Down
8	42	M	AG	Failure	Up	Down
9	76	M	AG	Success	Up	Down
10	63	F	AG	Success	Up	Down
11	58	F	AG, HG	Success	Up	Up
12	78	M	AG, GU scar	Success	Up	Down
13	71	F	AG	Success	Up	Down
14	65	F	AG	Success	Up	Down
15	57	M	AG	Success	Down	Down
16	78	F	AG, DU scar	Failure	Up	Down
17	74	M	AG	Success	Up	Down
18	51	F	AG	Failure	Up	Down
19	72	M	GU active	Success	Up	Down
20	79	M	AG	Success	Up	Down

AG, atrophic gastritis; B:F, Bacteroidetes:Firmicutes; DU, duodenal ulcer; F, female; GU, gastric ulcer; HG, haemorrhagic gastritis; M, male; RE, reflux esophagitis.

after eradication therapy (S3). On the day of sample collection, the patients were instructed to take a fresh stool in a test tube. Those samples were immediately sealed in a plastic bag containing a disposable oxygen-absorbing and carbon dioxide-generating agent, and then transported to the laboratory within several hours on ice. To count the number of anaerobic bacteria using the culturing method, fresh stool was spread over glucose blood liver (BL) agar plates containing 5% horse serum, and then incubated at 37°C for 72 hours in 10% H₂, 10% CO₂ and 80% N₂. The other samples were stored at -80°C until the 16S rDNA assay. For the extraction of DNA, faecal solids in the suspension were broken down using a FastPrep 24 Instrument (MP Biomedicals, Santa Ana, California, USA) with zirconia beads at 5 m/s for 2 min. Bacterial DNA extraction from the suspension was performed using a Magstration System 12GC (Precision System Science, Tokyo, Japan).

PCR amplification of the 16S rRNA gene and next-generation sequencing

The hypervariable V3-V4 region of the bacterial 16S rDNA was amplified by PCR with 341F¹¹ and R806¹² primers. PCR was performed in accordance with the method reported by Takahashi *et al.*¹³ Sequencing was conducted using a paired end and modified to 2×300 bp cycle run on an Illumina Miseq sequencing system (Illumina, San Diego, California, USA) with MiSeq Reagent Kit version 3 chemistry.

A 16S rDNA-based operational taxonomic unit (OTU) analysis

The paired-end reads for each sample were joined using the Fastq-Join and then processed with quality filtering.¹⁴ The number of quality filter passed reads were 984 296, 1 187 681 and 1 022 232 in the samples at S1, S2, and S3, respectively. Those high-quality reads were then sorted and grouped into OTUs using the Quantitative Insights Into Microbial Ecology pipeline¹⁵ with default settings. The reads were next clustered into OTUs at 97% pairwise identity. To analyse the bacterial composition, TechnoSuruga Lab Microbial Identification Database DB-BA 10.0 (TechnoSuruga Laboratory, Shizuoka-City, Japan) were used in accordance with the method reported by Hisada *et al.*¹⁶ In this composition analysis, we focused on

the genera representing >0.1% of the total microbiota, which was approximately >85% in this study. Hierarchical clustering based on the unweighted pair group method with arithmetic mean was performed using JMP V.8.0 (SAS Institute, Cary, North Carolina, USA) to analyse the overall bacterial community structure and the similarity in the frequency of identification of each taxon among the samples. The similarity between the bacterial communities was determined using Euclidean distance.

Statistical analyses

The paired t-test or Wilcoxon signed-rank test was used to compare the bacterial abundance and clinical laboratory data between different time points. The difference in the rate between groups was examined by Fisher's exact probability test. An analysis of similarity was used to test for statistical differences in bacterial community similarities using the vegan package (V.2.3-4) implemented in R (V.3.2.4). All P values were two sided and considered to be significant at P<0.05.

RESULTS

Patients with eradication therapy

A total of 20 patients who had been diagnosed with *H. pylori* infection and underwent eradication therapy participated in this study from April 2016 to March 2017. The mean age (SD) of them was 63.8 (13.2) years and male/female ratio was 10/10. Three patients whose ID numbers were 8, 16 and 18 were revealed to be in failure for eradication (table 1).

Change in the gut microbiota community structure after eradication therapy

The bacterial diversity within individual stool samples (alpha diversity) was evaluated using both the chao 1 index and the observed OTU number. Rarefaction curves showed no significant difference in the degree of diversity between before (S1) and 3 months after (S3) the eradication therapy (data not shown).

Alteration in the gut microbiota composition after eradication therapy

The number of anaerobes by the culturing method was marginally lower at S2 than at S1 but increased to

Table 2 Effect of eradication therapy on the number/abundance of bacteria

	Before (S1)	Eradication therapy		P value	Δ (S3-S1)
		Immediately after (S2)	3 months after (S3)		
Anaerobes by culturing method (×Log ₁₀ CFU/g)	10.11 (9.60–10.40)	9.65 (9.41–10.28)	10.10 (9.61–10.35)		0.76
Bacteroidetes:Firmicutes ratio (×100)	0.93 (0.30–3.31)	2.04 (1.40–10.62)	2.40 (1.40–7.84)		<0.01
Abundance (%)					
Genus Bifidobacterium	12.6 (7.6–23.2)	0.1 (0.006–5.275)	4.8 (0.3–17.1)		<0.01
Faecalibacterium prausnitzii	0.95 (0.12–2.18)	0.59 (0.006–4.54)	1.20 (0.10–2.83)		0.02
Akkermansia muciniphila	0.002 (0–0.288)	0 (0–0)	0 (0–0.69)		0.66

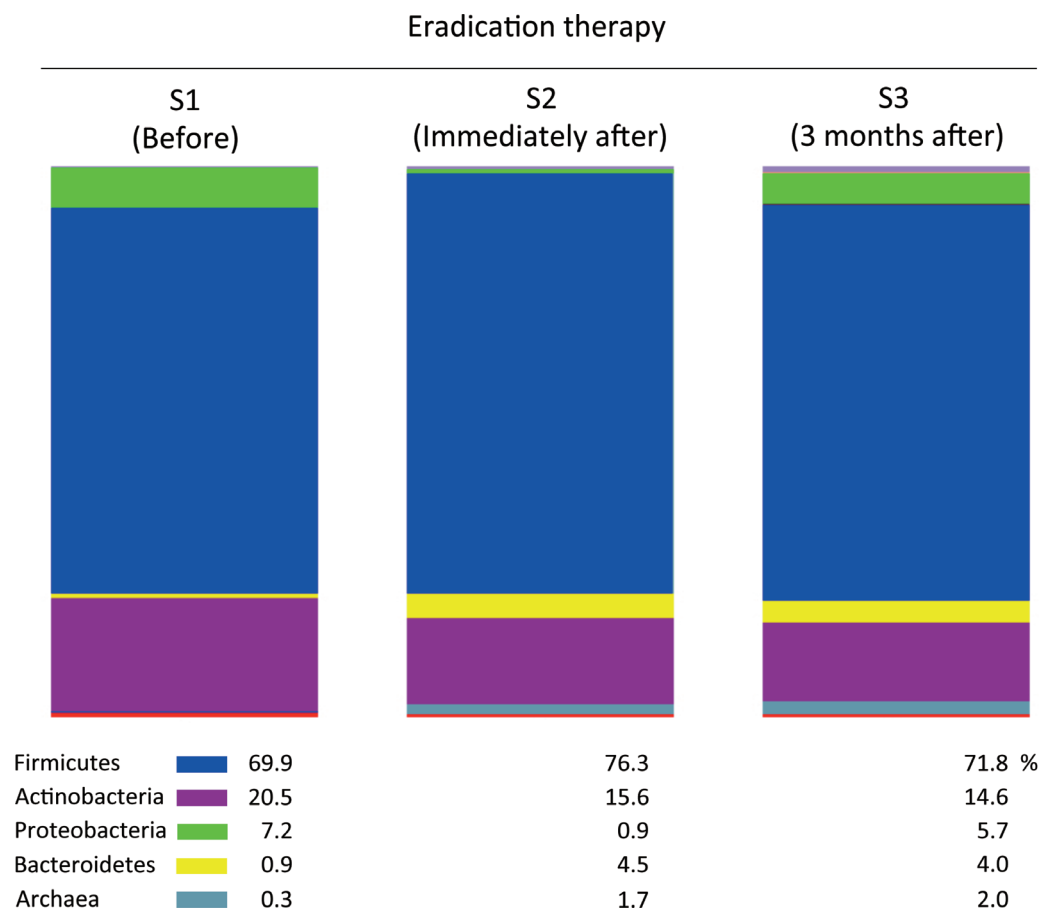


Figure 1 Proportion of the dominant five phyla in the faecal samples. The per cent proportions of the five dominant phyla of Firmicutes, Actinobacteria, Proteobacteria, Bacteroidetes and Archaea in all faecal samples at S1, S2 and S3 are depicted in the bar charts. The S1, S2 and S3 samples include 984 296, 1 187 681 and 1 022 232 high-quality reads, respectively.

the pretherapy level at S3 (table 2). The OTUs generated from the 16S reads were then classified into phyla according to a similarity search (figure 1). At S2, there was a marked increase in the abundance of both Bacteroidetes ((0.9% to 4.5%)) and Archaea but a decrease in that of both Actinobacteria ((20.5% to 15.6%)) and Proteobacteria. The increase in Bacteroidetes and decrease in Actinobacteria persisted even at S3 (0.9% to 4.0% and 20.5% to 14.6%, respectively). The abundance of Firmicutes, the predominant phylum, showed little change between S1 and S3 ((69.9% to 71.8%)). Thus, the median B:F ratio was significantly greater at S3 than at S1 ($P<0.01$, table 2). This increase in the B:F ratio was quite obvious, with 15 of the 20 patients (75%) showing a higher ratio at S3 than at S1 (table 1 and figure 2).

We then examined the abundance of particular bacteria considered to be associated with dysbiosis in obesity (table 2). The abundance of the genus *Bifidobacterium*, which is included in phylum Actinobacteria, was significantly lower at S3 than at S1 ($P<0.01$). In contrast, the abundance of *Faecalibacterium prausnitzii* was greater at S3 than at S1 ($P=0.02$). A double hierarchical clustering analysis demonstrated an opposite but closely related change in the abundance after therapy between *Bifidobacterium* and *Faecalibacterium* (figure 3), with a distinct cluster formed by Bifidobacteriaceae including

Bifidobacterium and Ruminococcaceae including *Faecalibacterium* in the analysis of similarity in the frequency of identification among samples in the clustering of bacterial families.

Change in the biomarkers regarding obesity/type 2 diabetes mellitus (T2DM) and their association with gut microbiota during the therapy

Among the clinical and laboratory data concerning obesity/T2DM examined in the present study (table 3), statistically significant decreases and increases were observed in the values of active ghrelin ($P=0.003$) and leptin ($P=0.03$), respectively, between S3 and S1. We therefore examined whether the change in the levels of those adipogenesis-regulating hormones was associated with the alteration of the ratio of B:F in each patient (figure 2). The rate of decrease in active ghrelin levels among patients with an increased B:F ratio was 14 out of 15, whereas the rate of decrease in active ghrelin levels among patients with a decreased B:F ratio was only 2 out of 5 (table 1). The difference in the rates between these two groups was statistically significant ($P=0.03$) by Fisher's test. No significant association was found between the change in the leptin level and the B:F ratio.

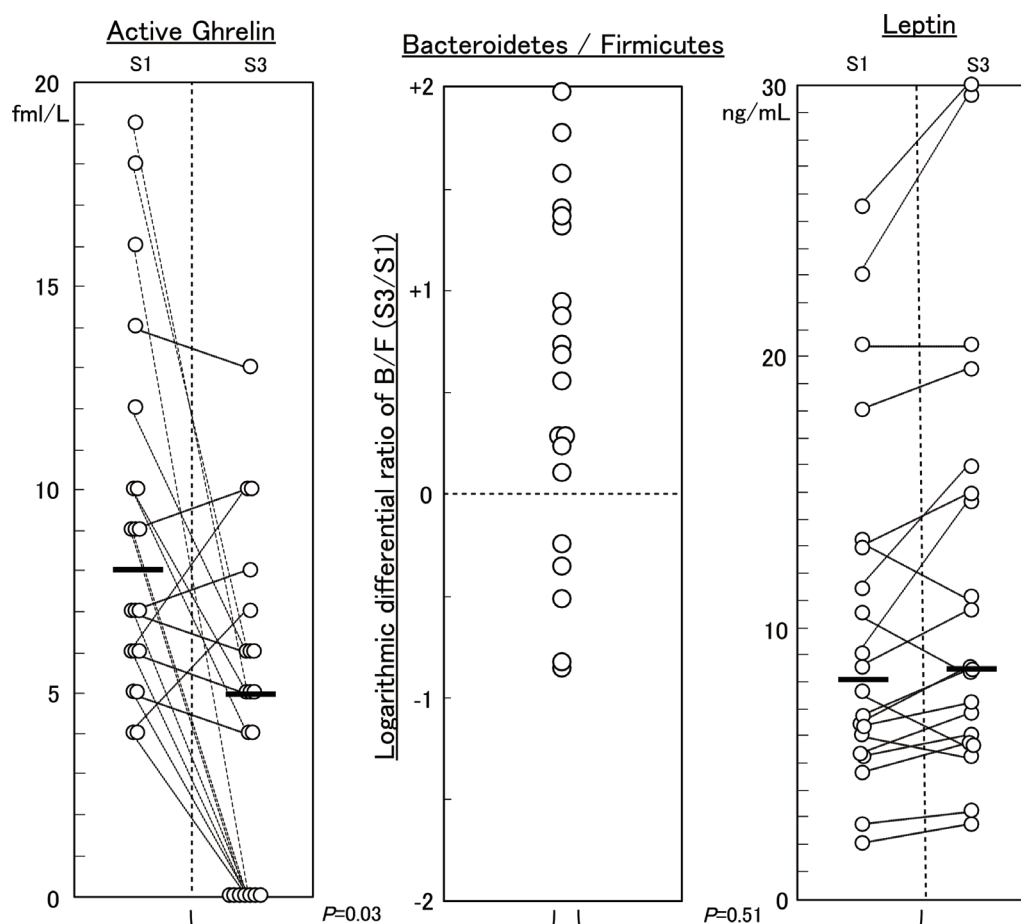


Figure 2 Difference in the bacterial ratios and hormone levels between S1 and S3. Each symbol represents the logarithmic differential ratio of Bacteroidetes:Firmicutes (B:F) (middle), the concentration of plasma ghrelin (left), and the concentration of serum leptin (right) in a patient at S1 and S3. Symbols representing the same patients are connected with lines. P values at the bottom indicate the statistical significance according to Fisher's exact probability test.

DISCUSSION

In recent studies of gut microbiota using high-throughput 16S rRNA gene sequencing, loss of diversity and marked short-term changes in several bacterial taxa have been a consistent finding after treatment with antimicrobials.¹⁷ In the present study using *H. pylori*-infected patients who were treated with first-line therapy using both amoxicillin and clarithromycin for 1 week, the alpha diversity in the stool samples showed no significant changes at 3 months after the therapy. Given that only a slight transient decrease in the number of bacteria was observed by a culturing method just after the therapy (see table 1), the antimicrobials used in the present study might have exerted only a moderate perturbing effect on the microbiota, thereby resulting in no marked decrease in the diversity of the gut microbiota after the therapy.

Of note, however, in the bacterial composition analysis at the phylum level, the B:F ratio significantly increased just after the therapy and remained increased even at 3 months after the therapy. Yap *et al*¹⁸ also reported that the alpha diversity was similar between pre-eradication and 6 months posteradication in stool samples obtained from *H. pylori*-infected young adults treated with amoxicillin (1000 mg twice daily) and clarithromycin

(500 mg twice daily), whose dose was pretty greater than that used in the present study. Those authors also observed not an increase but a decrease in the B:F ratio posteradication.

After external perturbation, such as antimicrobial treatment, the ecosystem of gut microbiota exhibits no change, resilience, adaptation or failing resilience.¹⁹ The robustness of the microbiota ecosystem is considered to be a major factor determining which of those responses the microbiota exhibit. The difference in the age and race between our subjects (Japanese, 42–79 years of age) and the subjects of Yap *et al* (Malaysian, 18–30 years of age) might induced in a difference in the robustness of the gut microbiota ecosystem, thereby resulting in different outcomes in the B:F ratio after antimicrobial treatment. In addition, differences in the dose of antimicrobials used for eradication and the duration of follow-up might also have affected the outcomes of these two studies. Furthermore, as the subjects without *H. pylori* infection were not included in our study, it is possible that some bias due to long-lasting *H. pylori* infection on the B:F ratio at S1 might be involved.

When analysing the existence of any similarities in the frequency of identification among samples (see figure 3),

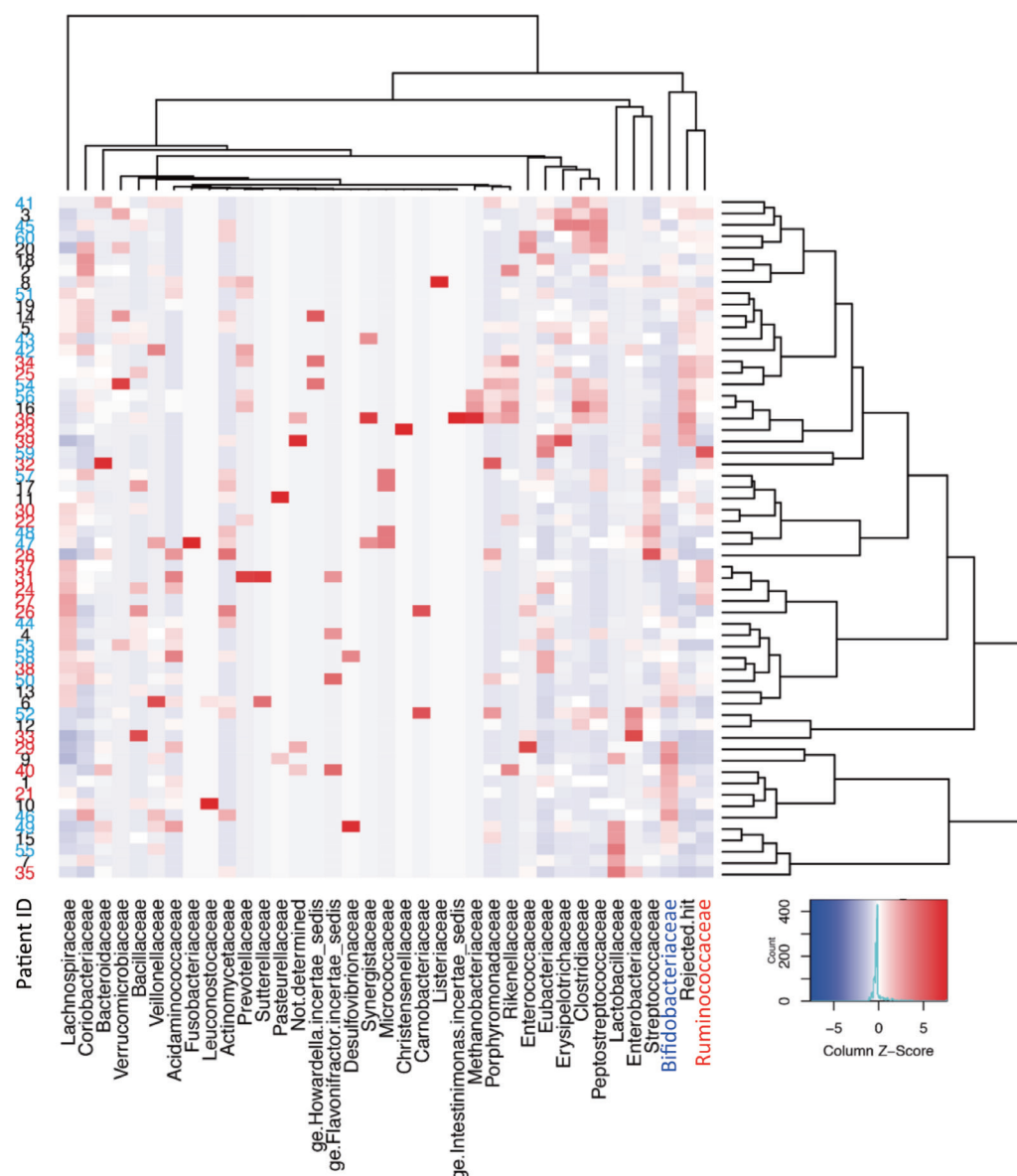


Figure 3 Double-hierarchical clustering analysis. The faecal samples from 20 patients at three time points for 36 dominant families are represented on a double-hierarchical clustering heat map. The clusters on the right side indicate similarity among the samples at the family level. Sample ID numbers 1–20, 21–40 and 41–60 on the left side represent those obtained at S1, S2 and S3, respectively. IDs 1, 21 and 41, and likewise 2, 22 and 42 and so on correspond to the same patient. The clusters at the bottom indicate the families showing similarity in the frequency of identification among samples. The Z-score column represents the relative ratio of abundance.

an opposite pattern of colonisation was found between *Bifidobacteriaceae* and *Ruminococcaceae* whose major genera were *Bifidobacterium* and *Faecalibacterium*, respectively. That is, a sample with a high density (=columns with red colour) of *Bifidobacteriaceae* contained a low density (=columns with blue colour) of *Ruminococcaceae*, and vice versa. This suggests that these two genera may share a common niche in the gut ecosystem. Thus, the administration of both genera together as probiotics may result in competition for nutrition and colonisation sites, despite their being considered beneficial bacteria for improving energy balance.^{20–21} Indeed, *Faecalibacterium* is known to be one of the major bacterial groups that produce butyrate in the human gut microbiota.²²

In the present study, the gut microbiota after the eradication therapy showed a phenotype not of ‘obesity’ but of ‘lean’, which was represented by an increase in both the B:F ratio⁹ and an abundance of *F. prausnitzii*.²² Furthermore, the plasma level of the active form of ghrelin was significantly lower after eradication therapy than before the therapy. A statistical analysis demonstrated a significant correlation between such changes in the B:F ratio and the ghrelin level. The majority of circulating ghrelin is produced in the gastric mucosa and stimulates the appetite, thus increasing the fat mass deposition and body weight.^{3–10} Therefore, in energy homeostasis, the increase in the B:F ratio in the gut microbiota was comparable with the decrease in the plasma ghrelin level. It has been reported that the plasma ghrelin

Table 3 Clinical and laboratory data regarding obesity/T2DM

	Eradication therapy		
	S1 (n=20)	S3 (n=20)	P value
Body weight (kg)	52.9 (51.6–70.2)*	53.9 (49.7–70.8)	0.35
Percent body fat (%)	26.7±8.3†	26.4±8.1	0.43
Body mass index	23.0±3.4	22.9±3.6	0.26
Serum triglycerides (mg/dL)	80.5 (69.8–139.3)	109.0 (65.8–155.3)	0.35
Fasting blood glucose (mg/dL)	105 (91–102)	108 (87–103)	0.99
Insulin (μIU/mL)	4.48 (2.62–6.81)	3.17 (1.86–9.56)	0.93
HOMA-R	1.02 (0.62–1.99)	2.05 (0.42–3.14)	0.78
HbA1c (NGSP %)	5.6 (5.3–6.0)	5.6 (5.4–5.9)	0.10
Adiponectin (μg/mL)	9.2 (7.1–12.8)	10.4 (6.5–14.1)	0.41
CRP (ng/mL)	511 (187–1083)	408 (202–1180)	0.18
Desacyl – ghrelin (fmol/L)	89.8±40.8	77.6±36.7	0.14
Active ghrelin (fmol/L)	8 (6–10.5)	5 (0–6.25)	0.003
Leptin (ng/mL)	8.1 (5.8–13.0)	8.5 (5.9–15.2)	0.03

*Median (IQR).

†Mean±SD.

CRP, C reactive protein; HbA1c, glycated haemoglobin; T2DM, type 2 diabetes mellitus. HOMA-R, homeostasis model assessment-insulin resistance.

level is regulated by multiple factors, including food intake, body weight and gastric ghrelin production.²³ Because the eradication of *H. pylori* is known to increase the production of ghrelin in the gastric mucosa,⁵ it appeared unlikely that the elimination of *H. pylori* caused the reduction in the plasma ghrelin level. Indeed, such reduction in the plasma ghrelin was observed even in the three patients in the present study who failed *H. pylori* eradication therapy. Therefore, some event other than the eradication of *H. pylori* itself may have caused the decrease in the plasma ghrelin level after eradication therapy in the present study, while it still needs further studies using sufficient number of *H. pylori*-infected subjects to exclude the *H. pylori* infection as a cause of the plasma ghrelin reduction.

Queipo-Ortuño *et al*²⁴ highlighted the association between the gut microbiota and appetite-regulating hormones using rat models with different nutritional conditions and levels of physical activity. In their study, plasma ghrelin level was significantly correlated with the quantity of several kinds of bacteria, including *Bifidobacterium* and *Bacteroides*. Also in the present study, both the plasma ghrelin level and the abundance of *Bifidobacterium*

significantly decreased in the patients after eradication therapy. While we still need to clarify the relationship between changes in the B:F ratio and the ghrelin level after the treatment with antimicrobials, changes in the gut microbiota, such as B:F ratio and abundance of both *Bifidobacterium* and *Faecalibacterium*, might cause a reduction in the plasma level of ghrelin, as the direct and earliest target of antimicrobials would be the microbiota, not the hormone-secreting system. Moreover, Lin *et al*²⁵ reported that butyrate, one of the major short-chain fatty acids formed by gut microbiota such as *Faecalibacterium*, whose abundance actually enlarged in the present study, reduced the plasma ghrelin level in the murine system. It has been reported that the gut microbiota significantly influence the host energy balance by affecting the energy yield from foods and modulating diet-derived compounds that alter the host metabolic pathway.⁸ Taken together, we suggested that ghrelin, an appetite-regulating hormone of stomach origin, might be a novel factor by which the gut microbiota affect the energy balance of the host. Furthermore, both the gut microbiota composition and the plasma ghrelin level might be used as an earliest biomarker to foresee the visceral obesity or insulin resistance.

Why such a 'lean' phenotype was found in both the gut microbiota composition and plasma ghrelin level at 3 months after the eradication therapy in the present study bears further investigation. A lower plasma ghrelin level at 3 months after the eradication therapy was also reported in Japanese patients in other studies.^{5,6} In contrast, however, a decrease in the B:F ratio, a phenotype of obesity, was observed at 6 months posteradication, which is 3 months later than the time-point for our assay.¹⁸ This suggests that an assay at 3 months after the termination of therapy might be too early to accurately assess the resilience of gut microbiota to reversely fluctuate towards a 'fat' phenotype in vulnerable patients, which may have increased the ghrelin level and then the body weight later. As such, the short duration of follow-up after the eradication therapy may be one limitation associated with the present study.

Contributors HY, AyT and MM recruited the *H. pylori*-infected patients undergoing eradication therapy and performed biochemical examinations. ST and GO performed bacteriological, genetical and statistical analyses. YK was involved in the study design and drafting the manuscript. AtT was involved in the study design and data interpretation.

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Competing interests None declared.

Ethics approval The ethics committee of Tokai University Hospital approved the study (15R-160).

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