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Original Article

Serum level of sex steroid hormone is associated with diversity and profiles of human gut microbiome

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ABSTRACT

Gut microbiota plays roles in host physiology including endocrine function. Although some data suggest a potential connection between biological sex differences and gut microbiota, the connection between sex steroid hormones and gut microbes remained unexplored. The current study investigates the relationship between gut microbes and serum levels of testosterone in men and estradiol in women. Fecal microbiota from a total of 57 men (n = 31) and women (n = 26) were assessed using 16s rRNA gene sequencing. Based on the levels of serum testosterone and estradiol in men and women, respectively, participants were stratified into three groups of Low, Medium, and High. Microbiome communities were analyzed as a function of the steroid hormone within sex. Men and women in the High group harbored more diverse gut microbial communities than others. In men, the abundance of *Acinetobacter*, *Dorea*, *Ruminococcus*, and *Megamonas* correlated significantly with testosterone levels. Women in the High group have more *Bacteroidetes* and less *Firmicutes* phyla than those in the Low group. Genera *Slackia* and *Butyricimonas* were significantly correlated with estradiol levels. These results demonstrate that sex steroid hormone levels are correlated with diversity and gut microbial composition, and provide fundamental information helpful for developing communication networks between human and microbial communities.

1. Introduction

The human body carries an enormous load of microorganisms which form a symbiotic relationship with the host. The gastrointestinal tract has the largest number of microbes in the human body, with the colonic microbial community in particular being the densest and most diverse [1]. Gut microbiota contribute to maintaining a series of functions in humans: they produce short-chain fatty acids (SCFAs) which can be energy substrates for gut epithelial cells, act as a supplier of vitamins to the host, and promote balance within the immune system [2]. Understanding what factors are responsible for shaping of gut microbiota has gained increasing attention over recent years. Intestinal microbiota in adults has been reported to be influenced by many factors including host genetics, obesity, age, disease state, diet, and other environmental variables, although the level of change may vary between individuals [3,4].

It has been shown that gut microbiota can function as an endocrine organ because they can produce and metabolize numerous chemicals with similarities to hormones [5]. Indeed, bacteria in the distal human ileum can affect the metabolism of bile acids by deconjugating primary bile acids bound to glycine, which decreases intestinal uptake of bile acids and makes them susceptible to further metabolism by gut microbiota [1]. SCFAs produced

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by intestinal microbes can have signaling functions and can induce receptor-mediated effects like leptin signaling [6,7]. Some bacterial strains have the ability to modulate the level of precursors for neurotransmitters, such as serotonin and dopamine, which affect the mood and behavior of the host [8,9]. In addition, gut microbiota can be involved in host metabolism, appetite, immunity and mating by influencing levels of various hormones including corticosterone, adrenocorticosterone, leptin, ghrelin and insulin [10]. Taken together, the crosstalk between gut microbiota and signaling molecules can affect human physiology, and efforts have continued to identify distinctive structures of the intestinal microbiome as they relate to specific hormone levels.

It has been suggested that gut microbiota can modulate sex hormones and their metabolites in the host [11,12]. Considering the fact that sex or gender differences are salient factors determining prominent features of host physiology and behavior, it is important to investigate whether the sex hormones have an interaction with intestinal microbes. It has been found that the composition of commensal microbiota of male mice was distinct from that of females at the time of puberty. Furthermore, they found that circulating testosterone levels increased after eliminating commensal microbiota in female mice [13]. Another study revealed the strong impact of gonadectomy and hormone replacement on the composition of microbiota in mice [14]. These observations implied that sex hormones have explicit effects on gut microbiota of the host. In addition, some cross-sectional studies have shown an association between gut microbiota and systemic levels of estrogens. It was reported that women whose gut microbiota was more diverse had elevated ratios of hydroxylated estrogen metabolites to estradiol in their urine [15]. Another cross-sectional study also identified a correlation between levels of urinary estrogens, and the richness and diversity of the fecal microbiome. Moreover, systemic estrogens had a strong positive correlation with fecal *Clostridia* taxa and specific genera in the *Ruminococcaceae* family [16]. It has been proposed that estrogens conjugated in the liver are usually excreted from the body via the urine or with bile in the feces. However, the conjugated estrogens can be transformed by specific bacterial genera colonizing the human intestine, which have active beta-glucuronidase. This increases the reabsorption of estrogens into the blood and lowers their elimination from the body [17]. These results indicate that systemic levels of estrogens and their metabolites could be influenced by specific genera of the gut microbiome.

Although some previous studies showed the interactions of the hormones with gut microbes, the vast majority of them have been carried out with animal models [18,19]. Also, there are only a few human studies which have examined the changes in the bacterial communities by the sex steroid hormone [16,20], and the results remain controversial in some murine studies [21,22]. Furthermore, most studies have focused on estrogen [15,16,18,20,23,24], and only a few studies reported the association of intestinal microbial composition to levels of testosterone [25,26]. Therefore, to investigate the association of human intestinal microbiota with the levels of sex steroid hormone, we assessed the serum levels of testosterone in men and the levels of estrogen in women, and conducted 16rRNA pyrosequencing for the profiling analysis of their gut microbiome. In addition, we aimed to discover the specific bacterial taxa that correlate with the levels of serum estradiol or testosterone.

2. Materials and methods

2.1. Participants

A total of 57 participants between 25 and 65 years were enrolled in this study. Participants that regularly consumed dietary supplements, lipid-lowering and glucoregulatory medication, pre- or probiotics, or antibiotics within 6 months prior to sampling were excluded. Individuals were also excluded if they had gastrointestinal disorders, cancer, inflammatory diseases, diabetes or active heart disease. All aspects of this study were approved by Seoul National University Institutional Review Board (IRB No. 1506/002-014). All participants provided written informed consent prior to participating in the study.

2.2. Anthropometric and biochemical parameters

Height and body weight were measured to the nearest 0.01 cm and 0.01 kg using stadiometer and Inbody 4.0 (Biospace Co. Ltd, Seoul, Korea) respectively. Body mass index (BMI) was calculated as body weight divided by the height squared (kg/m^2). Fasting blood (8 h minimum) samples were collected in Serum Separator Tube (SST) and allowed to clot for 30–40 min. Serum was separated from the clot by centrifugation (3000 rpm, 10 min). Testosterone levels were measured in serum samples using a radioimmunoassay kit (Asbach Medical Products, Obrigheim, Germany) and read on a gamma counter device (Corba 5010 Quantum, PACKARD, USA). Serum estradiol levels were determined by an electrochemiluminescence immunoassay (ECLIA) using Modular Analytics E170 (Elecsys module) immunoassay analyzers (Roche Diagnostics, Mannheim, Germany). Measurements of serum testosterone and estradiol were done by Green Cross LabCell (GCLC) corp. (Yongin, Korea).

2.3. Stool collection and genomic DNA extraction

After providing informed consent, participants were asked to collect fecal samples at home. They were mailed specimen collection kits that included a freezer pack and a barcoded container with illustrated instructions for collecting specimens. After fecal collection, the fresh samples were shipped directly to our laboratories on pre-frozen freezer packs within 2 h and stored at −80 °C until DNA extraction. Total bacterial DNA was isolated from fecal samples using the QIAamp® DNA stool Mini Kit (QIAGEN, Hilden, Germany) according to the kit protocol for pathogen detection with a few modifications. Briefly, an average of 200 mg of fecal material was homogenized with 5 mm sterilized steel bead in ASL buffer using TissueLyser (QIAGEN, Hilden, Germany) for 1 min at 30 Hz. We then heated the suspension at an optional high-temperature (95 °C) to lyse gram-positive bacterial cells. In the last incubation step, we increased the elution time from 1 min to 5 min to increase DNA yield.

2.4. Sequencing of 16s rRNA genes

Bacterial 16S rRNA gene amplicons were amplified with universal primers, which anneal to the hypervariable region V1–V2 of the bacterial 16S rRNA gene as described previously [27]. A list of the sequences of these fusion primers is provided in the supplementary table (Table S1). After quantification by BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA), fifty pooled libraries were enriched and loaded on Ion 530™ chip by Ion Chef™ (Thermo Fisher Scientific, Waltham, MA). Libraries were then sequenced by an Ion S5™XL platform (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions.

2.5. 16s rRNA sequencing data analysis

The sequencing data were analyzed using QIIME 1.9.1. First the sequences were demultiplexed and quality filtered using split_libraries.py command with default variables (sequence length

between 200 bp and 1000 bp; minimum average quality score 25; no primer mismatch allowed). Putative chimeric sequences were identified and eliminated in the quality-filtered sequences with the scripts 'identify_chimeric_seqs.py' and 'filter_fasta.py' using USERCH 6.1. The joined high quality sequences were assigned as operational taxonomic units (OTUs) at 97% sequence similarity against the Greengenes 16S rRNA gene database (version 13_8). This was performed using UCLUST-based open-reference OTU picking workflow. Relative abundance of taxa from domain to genus was assigned using the summarize_taxa.py command. Microbial diversity was calculated within samples (α-diversity) using Chao 1 richness, Simpson evenness, Good's coverage and Shannon diversity.

2.6. Statistical analyses

The data were not normally distributed so we used nonparametric tests to perform a statistical evaluation. For comparisons between two independent groups, Mann–Whitney test was used. Differences between more than two groups were evaluated by Kruskal–Wallis test with Dunn's multiple comparison post hoc test. Associations between variables were tested using Spearman's rank correlation coefficient. All statistical analyses was performed using GraphPad Prism version 6 (GraphPad Software, Inc, La Jolla, CA) and the statistical software SPSS version 23 (SPSS Inc., Chicago, Illinois, USA).

3. Results

3.1. Classification of participants based on their serum sex hormone levels

The fifty seven participants consisted of 31 men and 26 women. Their age, height, weight and BMI are presented in Table 1. Participants were stratified into three categories based on the serum levels of testosterone or estradiol in men and women, respectively. Men were grouped into three categories as having Low (tertile1; <3.55 ng/ml), Medium (tertile2; 3.55–4.55 ng/ml) and High (tertile3; >4.55 ng/ml) level of serum testosterone (Table 1 and Supplementary Fig. S1a). In the same manner, women were divided into three subgroups by estradiol levels: Low (tertile1; <5.0 pg/ml), Medium (tertile2; 5.0–60.0 pg/ml), and High (tertile3; >60.0 pg/ml) (Table 1 and Supplementary Fig. S1b). No statistically significant differences in age, height, weight, and BMI were observed among the Low, Medium and High testosterone groups in men. In women, expectedly, those in the High estradiol group were significantly younger than those in the Low group (P = 0.0076). Except for age, there were no significant differences in height, weight and BMI among the three groups of women (Table 1).

3.2. Gut microbiota profiling analysis of men and women

Sequencing analysis of 16S rRNA gene in fecal samples from all participants yielded a total of 16,590,992 high quality sequences (291,070 ± 64,609 reads/fecal sample, mean ± SD). These sequences were mapped to 3272 ± 767 OTUs (range = 1235–4904) by an open-reference OTU picking algorithm. We first calculated the Good's coverage index, which accounts for sequencing depth, to assess whether the sequences sufficiently characterize the microbial composition. Good's coverage averaged 0.986 ± 0.004 (range = 0.975–0.985) for individual samples (Fig. 1a). Because the average value of Good's coverage reached to 0.99 for all sequences in the individuals, the results were sufficient to investigate gut microbial communities in the subjects.

Next, diversity, richness and evenness of bacterial community was examined and compared between male and female. Diversity (Shannon index; women: 6.064 ± 0.166; men: 5.959 ± 0.150; p = 0.778), richness (Chao1 index; women: 6857 ± 308.7; men: 6841 ± 335.8; p = 0.976), and evenness (Simpson's Evenness index; women: 0.0071 ± 0.0009; men: 0.0068 ± 0.0006; p = 0.693) were not significantly different between women and men (Fig. 1b). We investigated further whether the relative abundance of specific taxa might differ between male and female. At the phylum level, no specific phyla were identified as significantly different between two sex groups (Fig. 1c and Supplementary Fig. S2). The abundances of *Bacteroidetes* and *Firmicutes* that make up the vast majority of the gut microbial community in human gut (>95%) were not significantly different between men and women, and the ratios of *Firmicutes* to *Bacteroidetes* were also similar (Fig. 1d). These data imply that two dominant microbial phyla of gut microbiota were independent of sex. Although several animal studies reported sex differences in gut microbiota [13,28,29], it has been established that studying sex-related associations of the gut microbial community in humans is more difficult than for animal studies [29–31]. This is because comparisons of human males and females is influenced not only by biological sex but also by cultural gender-related factors. Although the gut microbiota interplay with various socio-environmental factors in humans, it is critical to elucidate their interaction with the host genome and biological factors separate from non-biological factors. In keeping with the concept that sex steroid hormones plays important roles in defining biological sex, we extended our studies to investigate the gut microbiota signature associated with sex hormones.

3.3. Sex steroid hormones influence the diversity of the gut microbiome

To address the question of whether sex hormones affect the bacterial communities, we examined the richness, evenness and

Table 1
Characteristics of study population as a whole by tertiles of serum testosterone (men) and estradiol (women) levels.

Characteristics	Total (n = 31)	Men			P-value[a]	Total (n = 26)	Women			P-value[a]
		Levels of serum testosterone concentration					Levels of serum estradiol concentration			
		Low (<3.55 ng/ml) (n = 10)	Medium (3.55–4.55 ng/ml) (n = 11)	High (>4.55 ng/ml) (n = 10)			Low (<5.0 pg/ml) (n = 9)	Medium (5.0–60.0 pg/ml) (n = 8)	High (>60.0 pg/ml) (n = 9)	
Age	37.45 ± 1.91	38.70 ± 3.37	36.82 ± 2.61	36.90 ± 4.25	0.8389	46.15 ± 2.26	54.89 ± 1.03[a]	44.00 ± 4.93[ab]	39.33 ± 3.23[b]	0.0076
Height	174.14 ± 0.85	172.90 ± 1.41	176.00 ± 1.60	173.40 ± 1.31	0.3232	157.49 ± 1.13	156.90 ± 1.72	158.30 ± 2.21	157.40 ± 2.15	0.8425
Weight	84.35 ± 1.85	85.61 ± 3.51	87.39 ± 3.48	79.74 ± 2.14	0.4178	66.93 ± 2.41	61.50 ± 2.22	67.58 ± 3.38	71.78 ± 5.65	0.2253
BMI	27.78 ± 0.52	28.64 ± 1.09	28.18 ± 0.96	26.48 ± 0.43	0.3381	26.91 ± 0.85	24.90 ± 0.47	26.88 ± 0.87	28.94 ± 2.15	0.1503

Differences were considered significant at p < 0.05. Data are presented as mean ± SEM.
[a] P-value is for the comparison of all subject groups across estradiol (women) or testosterone (men) levels using Kruskal–Wallis test followed by a Dunn's multiple comparisons test.

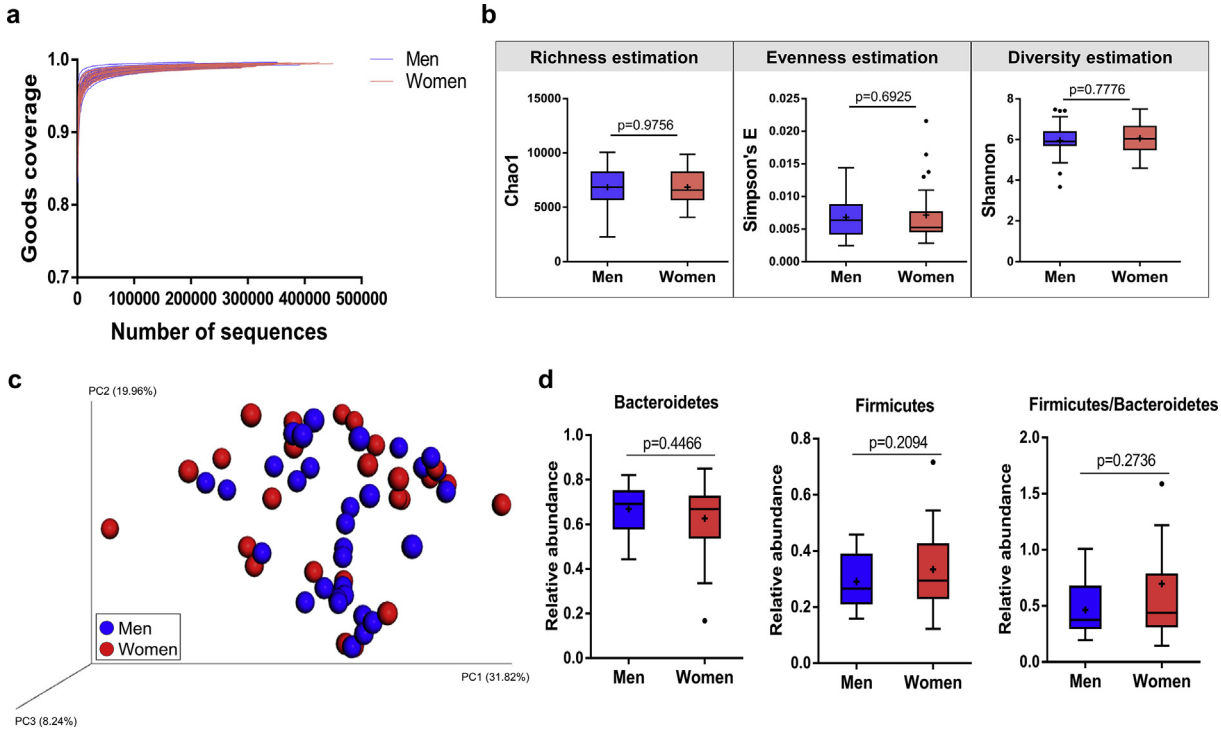

Fig. 1. Comparison of fecal microbiota between men and women. **a** Rarefaction curves based on the good's coverage indices. **b** Alpha-diversity indices for gut microbial richness (Chao1), evenness (Simpson's E), and diversity (Shannon) between men and women. **c** Principal coordinate analysis (PCoA) plot of bacterial community composition in men (blue) and women (red) using weighted UniFrac metric at the OTU level. Each sphere represented the fecal microbiota of a participant. **d** The relative abundance of *Firmicutes*, *Bacteroidetes*, and *Firmicutes* to *Bacteroidetes* ratio in the indicated groups. The box plots show the interquartile range (IQR), from 25th and 75th percentile, and the heavy horizontal line crossing the box represents the median values. Whiskers in the box plots denote minimum to maximum values. The circles represent outliers beyond the whiskers. Two-tailed Mann–Whitney rank-sum test was used to determine significance in men and women individuals.

diversity of gut microbiota in the groups Low, Medium and High for testosterone or estradiol. No significant changes in the richness determined by Chao1 were found among the groups of Low, Medium and High levels of testosterone or estradiol (Fig. 2a, b). In comparisons of diversity and evenness, however, the High testosterone group shows significantly higher scores than the medium group in men ($p = 0.0462$, Shannon index; $p = 0.0408$, Simpson's E index; Fig. 2a). Likewise in women, the High estradiol group exhibits higher diversity and evenness scores than the Medium group ($p = 0.0245$, Shannon index; $p = 0.0128$, Simpson's E index; Fig. 2b). These data suggest that men and women in the highest tertiles have more diversity and evenness of their gut microbial community.

3.4. Identification of testosterone-responsive intestinal bacteria in men

To study gut microbiota profiles that change in association with blood levels of testosterone, the abundance of each taxon was compared among the Low, Medium and High groups. The present study identified 11 bacterial phyla in the fecal samples - *Verrucomicrobia*, *Synergistetes*, *Bacteroidetes*, *Lentisphaerae*, *Firmicutes*, *Tenericutes*, *Fusobacteria*, *TM7*, *Proteobacteria*, *Cyanobacteria*, and *Actinobacteria* - and their relative abundances were presented for each group (Fig. 3a). No significant phylum- and family-level taxon difference was found in the comparison of the three groups. At the genus level, however, we found significantly enriched microbial taxa in the High compared to the Low group. While *Atopobium* was decreased, *Acinetobacter*, *Dorea*, *Ruminococcus*, *and Megammonas* were increased in the High testosterone group (Fig. 3b). Next, correlation analyses between the relative abundance of each genera and the level of hormone in all participants were performed. It is of interest that *Acinetobacter*, *Dorea*, *Megammonas* and *Ruminococcus* were positively correlated with the level of serum testosterone. *Ruminococcus* in particular turned out to be the most strongly responsive to the level of the hormone ($p = 0.0094$; Fig. 3c).

3.5. Estradiol modulates the ratio of *Bacteriodetes* to *Firmicutes* in the gut

The same 11 phyla identified in men were also found in women. Their abundances in each group of Low, Medium and High estradiol were then compared (Fig. 4a). The dominant components of the gut bacterial phyla were *Bacteroidetes* ($62.90 \pm 8.18\%$, mean ± SD), *Firmicutes* ($33.55 \pm 7.30\%$), followed by *Proteobacteria* ($3.30 \pm 0.78\%$) in all samples (Fig. 4a). Despite exhibiting broad distributions within a group (Fig. 4a), the relative abundances of *Bacteroidetes* were increased in association with the levels of estradiol with those of *Firmicutes* being conversely decreased (Fig. 4b). Accordingly, the ratio of *Firmicutes* to *Bacteriodetes* decreased with increasing levels of the hormone (1.2 in Low, 0.6 in Medium and 0.4 in High; Fig. 4b).

Analysis at the family level identified 9 families in the phylum of *Bacteroidetes* and 25 families in the *Firmicutes*. Within the *Bacteroidetes* phylum, *Bacteroidaceae* (61.2%), *Prevotellaceae* (28.6%), and *Rikenellaceae* (3.6%) dominated all groups. The relative abundance of *Bacteroidaceae* tended to increase, while *Rikenellaceae*, *Porphyromonadaceae*, and *Odoribacteraceae* were lower in the High estradiol group than the Low estradiol group (Fig. 4c). In the *Firmicutes* phylum, the predominant families were *Ruminococcaceae* (42.3%), *Lachnospiraceae* (39.9%), and *Veillonellaceae* (11.7%). The relative abundance of *Veillonellaceae* tended to increase, while

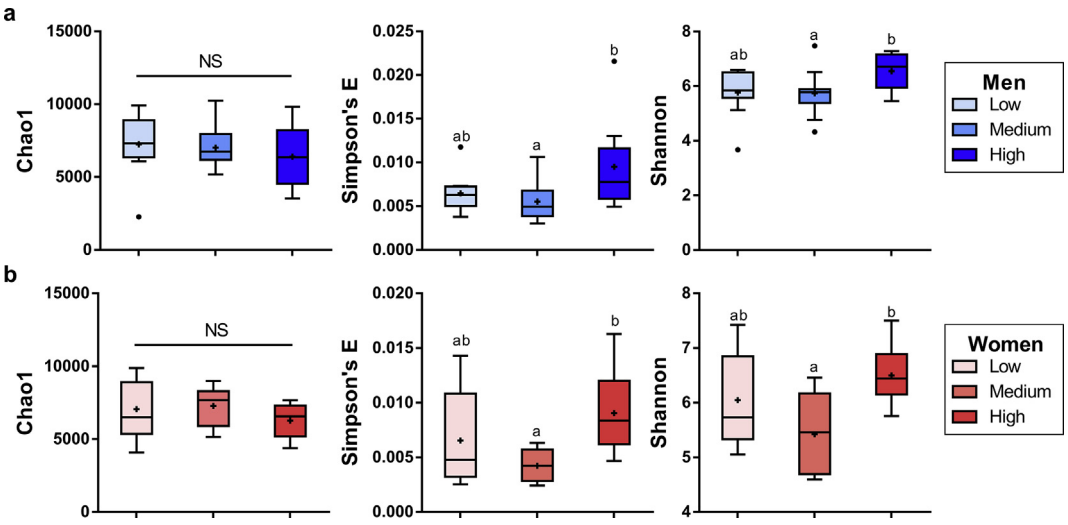

Fig. 2. Alpha diversity (Shannon), richness (Chao1), and evenness (Simpson's E) variation in male and female gut microbiota in response to serum sex steroid hormone levels. **a** Alpha diversity (Shannon), richness (Chao1), and evenness (Simpson's E) variation in men gut microbiota by serum testosterone levels (Low, Medium, High). **b** Alpha diversity (Shannon), richness (Chao1), and evenness (Simpson's E) variation in women gut microbiota by serum estradiol levels (Low, Medium, High). The box plot show the interquartile range (IQR), from 25th and 75th percentile, and the heavy horizontal line crossing the box represents the median values. Whiskers in the box plots denote minimum to maximum values. The circles represent outliers beyond the whiskers. Samples were analyzed using a Kruskal–Wallis test followed by a Dunn's multiple comparisons test ($P < 0.05$). Different lowercase letters indicate statistical significant differences in median value among groups by Dunn's test. NS indicates that the values are not significant at $P < 0.05$.

Lachnospiraceae tended to be lower in the High than the Low estradiol group, although this tendency was not statistically significant (Fig. 4d).

3.6. Identifying bacterial genera associated with serum estradiol concentration

Next, we sought to identify the specific bacterial genera that discriminated the gut microbiomes of estradiol Low from those of estradiol High women. To address this issue, we compared the relative abundance of gut microbial genera between the Low and High estradiol groups of women. Only 8 of the 133 genera were identified as differentially enriched between the Low and High estradiol groups. Compared to the High estradiol group, stool of the Low estradiol group was depleted in the bacterial genera *Veillonella*, but enriched in the genera *Slackia*, *Lactococcus*, *Christensenella*, *Dehalobacterium*, *Adlercreutzia*, *Odoribacter*, and *Butyricimonas* (Fig. 5a).

Then, we assessed the correlation between serum estradiol levels and the specific 8 genera. The relative abundances of *Slackia*, *Butyricimonas*, *Christensenella*, and *Dehalobacterium*, were significantly and negatively correlated with the levels of serum estradiol ($P < 0.05$; Fig. 5b). Because serum estradiol levels are closely associated with age in women, ($r = -0.70$, $p < 0.0001$; data not shown), we excluded the genera that are significantly correlated with age - *Christensenella* and *Dehalobacterium* (Supplementary Fig. S4). Therefore, these data suggest that the drop in serum estradiol concentration in women could be accounted for by the relative overabundance of *Slackia* ($r = -0.4200$, $p = 0.0327$) and *Butyricimonas* ($r = -0.3935$, $p = 0.0467$). Although a causal relation between gut microbiota composition and serum estradiol levels needs to be explored further, this finding suggests that the relative abundance of *Slackia* and *Butyricimonas* closely correlated with serum estradiol levels, even when the influence of age was excluded.

4. Discussion

To our knowledge, this is the first *in vivo* human study to investigate the relationships between serum levels of sex steroid hormones and the composition of intestinal microflora in both male and female participants using 16s rRNA sequencing methods. Our study showed that gut microbiome diversity was associated with the levels of testosterone in men and estradiol in women, although no sex differences were found in gut microbial diversity and composition. In addition, there were different patterns across sex in gut microbial communities related to sex steroid hormone levels. A number of gut microbial genera are differentially associated with serum testosterone levels in men or estradiol concentrations in women. These results show that serum sex steroid hormone concentrations are linked to changes in the gut microbiome.

It is well understood that men and women exhibit differences in body composition, the incidence of metabolic disease and even gastrointestinal transit time [32]. The mechanisms that underlie these disparities in physiology between genders are not clearly understood, but sex steroid hormones, such as the female sex hormone estradiol and the male sex hormone testosterone, might be considered dominant factors [13]. In this context, it is tempting to speculate that differences in the composition of gut microbiota between men and women are also likely to be of significant importance. However, in the current data set, there were no statistically significant differences between the microbiomes of men and women at the phylum level. Our findings are similar to those of studies [33,34], which found that there were no differences of gut microbial profiling between men and women. In contrast, our results are inconsistent with the previous study that reported females showed a lower abundance of Bacteriodetes compared to males [35]. This discrepancy from our findings might be due to the difference in the study population. Another factor that may contribute to this inconsistency is the fact that sex differences are influenced by socio-cultural as well as biological factors. In humans, gender, which refers to social-environmental factors related to being a man or a woman, also impacts the health and disease of individuals [36]. The social factors such as income, education level, occupation, and lifestyle have been claimed to affect human physiological characteristics [37,38]. Testosterone levels in particular were found to be influenced by social status. For instance, wielding power and competition can increase testosterone levels in women [39] and men [40]. Hence, future research should also be directed at

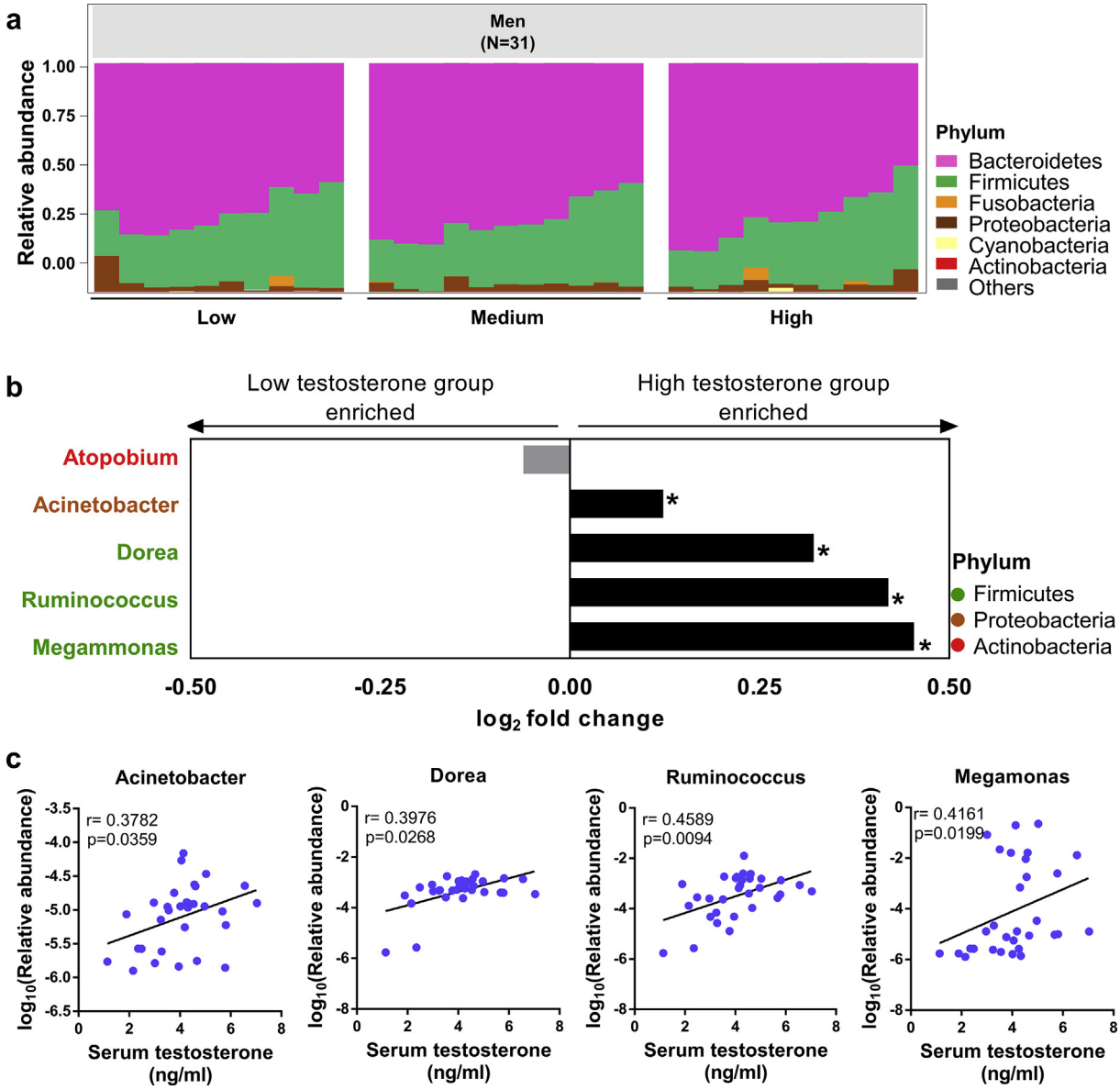

Fig. 3. A comparison of gut microbiome taxonomic profiles in serum testosterone level groups of men. **a** The relative abundances of the various bacterial phyla in each sample among each group of men (n = 10–11). Each bar represents one sample; colors depict the relative abundance of a particular phylum in each sample. Only phyla with ≥0.1% mean relative abundance are shown. **b** Five most abundant genera that differ significantly between the Low and High testosterone group in men as determined by the $\log_2$ of the fold change between two groups. The High testosterone group with enriched taxa are indicated with a positive $\log_2$ fold change (black), and taxa enriched in the Low testosterone group have a negative value (grey). Asterisk represents statistical significance ($P < 0.1$; two-tailed Mann–Whitney U test). **c** Correlation between serum testosterone levels and relative abundance of four genera (*Acinetobacter*, *Dorea*, *Ruminococcus*, and *Megamonas*) in men (N = 31). Linear regression is shown, and the Spearman correlation coefficient (r) and P-value are indicated.

understanding the relationships between gender (in addition to sex) and the gut microbiome.

It is notable that many previous studies have demonstrated that obesity is related to specific microbiota pattern characterized by an increase in the ratio of *Firmicutes* to *Bacteroidetes* (F/B ratio) [41]. Ruth E. Ley et al. showed that obese people had the relatively fewer *Bacteroidetes* and more *Firmicutes* compared with lean people, and the dietary weight loss resulted in an decrease in F/B ratio [42]. Similarly, the research conducted with Japanese population demonstrated that the F/B ratio was significantly higher in the fecal samples obtained from the obese subjects compared to those from non-obese individuals [43]. However, it is still ambiguous to draw a definite conclusion that obesity alone contributes to making the distinctive pattern of microbiota as some contradictory results also have been observed [44,45]. In the present study, our data showed a significant increase in the F/B ratio in the Low estradiol group as compared to the High estradiol group. It has been suggested that a higher F/B ratio can influence the energy harvest from the diet, and thus be associated with obesity [46]. Estradiol can regulate food intake, adipose tissue distribution, and energy balance, and the loss of estrogen with menopause is associated with an increase in central fat [47]. Thus, the F/B ratio accumulated in intestinal microorganisms of the Low estradiol group allows us to speculate that the interconnection between gut microbiota and low serum estradiol levels might result in energy accumulation. In fact, through prediction analysis for functional content from bacterial community, we observed the significant difference in Lipid metabolism between Low vs. High estrogen group (Supplementary

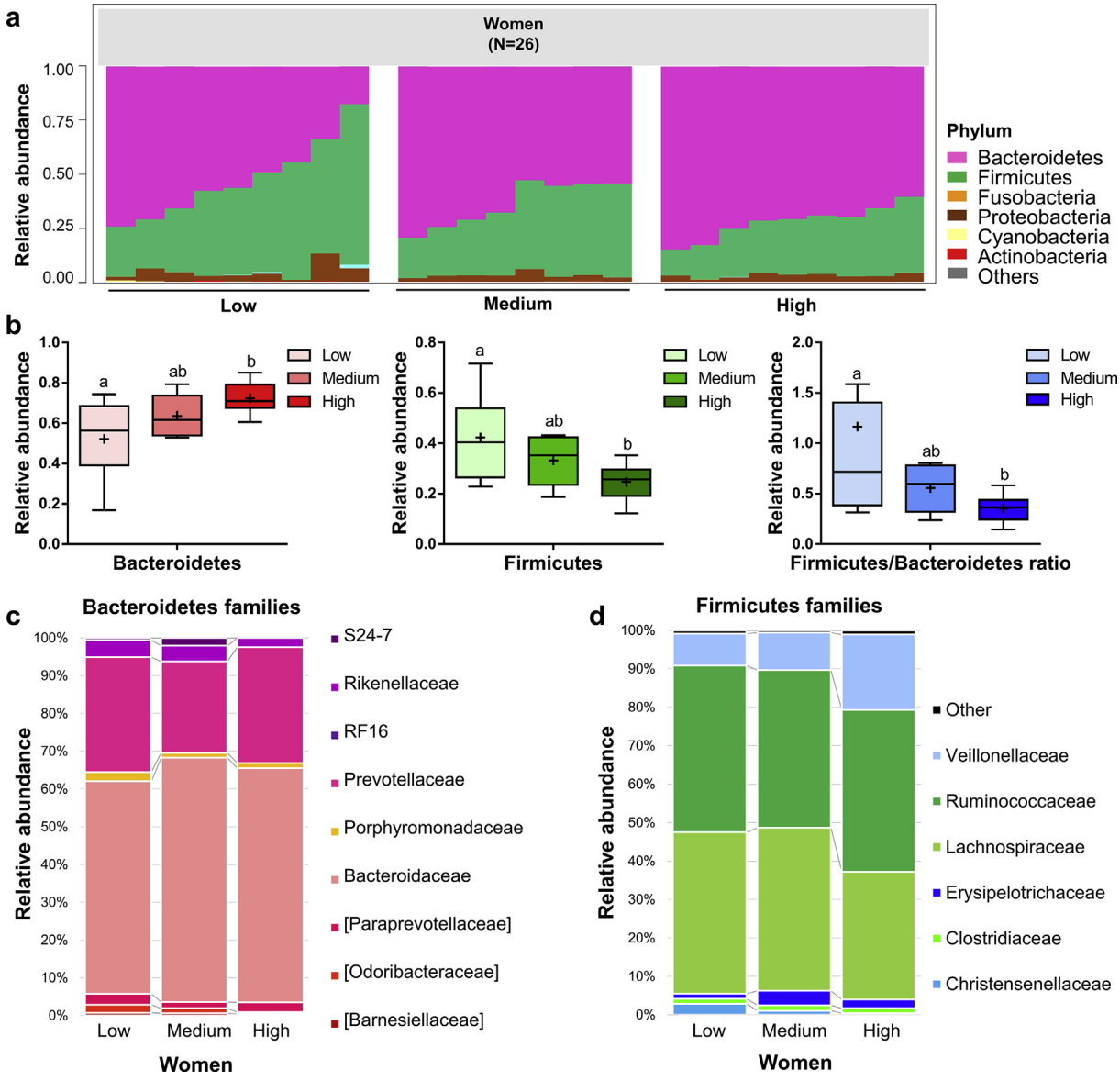

Fig. 4. A comparison of gut microbiome taxonomic profiles in serum estradiol level groups of women. **a** The relative abundances of the various bacterial phyla in each sample among each group of women (n = 8–9). Each bar represents one sample; colors depict the relative abundance of a particular phylum in each sample. Only phyla with ≥0.1% mean relative abundance are shown. **b** Depiction of the two predominant bacterial phyla, *Bacteroidetes*, *Firmicutes*, and *Firmicutes* to *Bacteroidetes* ratio, in three groups of women. The box plot shows the interquartile range (IQR), from 25th and 75th percentile, and the heavy horizontal line crossing the box represents the median values. Whiskers in the box plots denote minimum to maximum values. The circles represent outliers beyond the whiskers. Samples were analyzed using a Kruskal–Wallis test followed by a Dunn's multiple comparisons test. Different lowercase letters indicate statistical significant differences in median value among groups by Dunn's test ($P < 0.05$). **c,d** Average relative abundances of the most predominant bacterial families within the (**c**) *Bacteroidetes* and (**d**) *Firumicutes* in Low, Medium, and High estradiol groups of women (n = 8–9). Data are mean values. Only families with ≥0.001% mean relative abundance are shown.

Fig. S5). Additionally, this results suggest that the F/B ratio can be affected not only by BMI or weight, but also biological conditions including serum estradiol concentrations in women.

In women, serum estradiol levels were associated with relative abundances of taxa at both the phylum and genus levels. A high estradiol level was associated with an altered gut microbial community associated with increased and reduced contents of the phyla *Bacteroidetes* and *Firmicutes*, respectively. These effects of estradiol were independent of the individual's enterotype, because each relative abundance of *Bacteroides*, *Prevotella*, or *Ruminococcus* that is known to be major classifier in enterotyping was not significantly associated with serum estradiol level in women (Supplementary Fig. S3). This finding is different from a previous animal study that found that the relative abundance of *Bacteroidetes* was significantly increased in an ovariectomized rat model [48]. At the genus level, we observed a significant increase in the number of *Slakia* and *Lactococcus* in the estradiol Low group as compared to the High estradiol group, although it is unclear if the changes are driven by estradiol per se, or the other myriad changes associated with menopause. *Slakia* and *Lactococcus* have the capacity to metabolize dietary isoflavone daidzein into equol or dihydrodaidzin [49]. Equol are estrogen-like compounds that also have health benefits in relation to obesity, diabetes, and breast cancer [50,51]. Additionally, the Low estradiol group had a higher abundance of butyrate-producing bacteria, like *Butyricimonas*, compared to High estradiol group. These results imply that serum

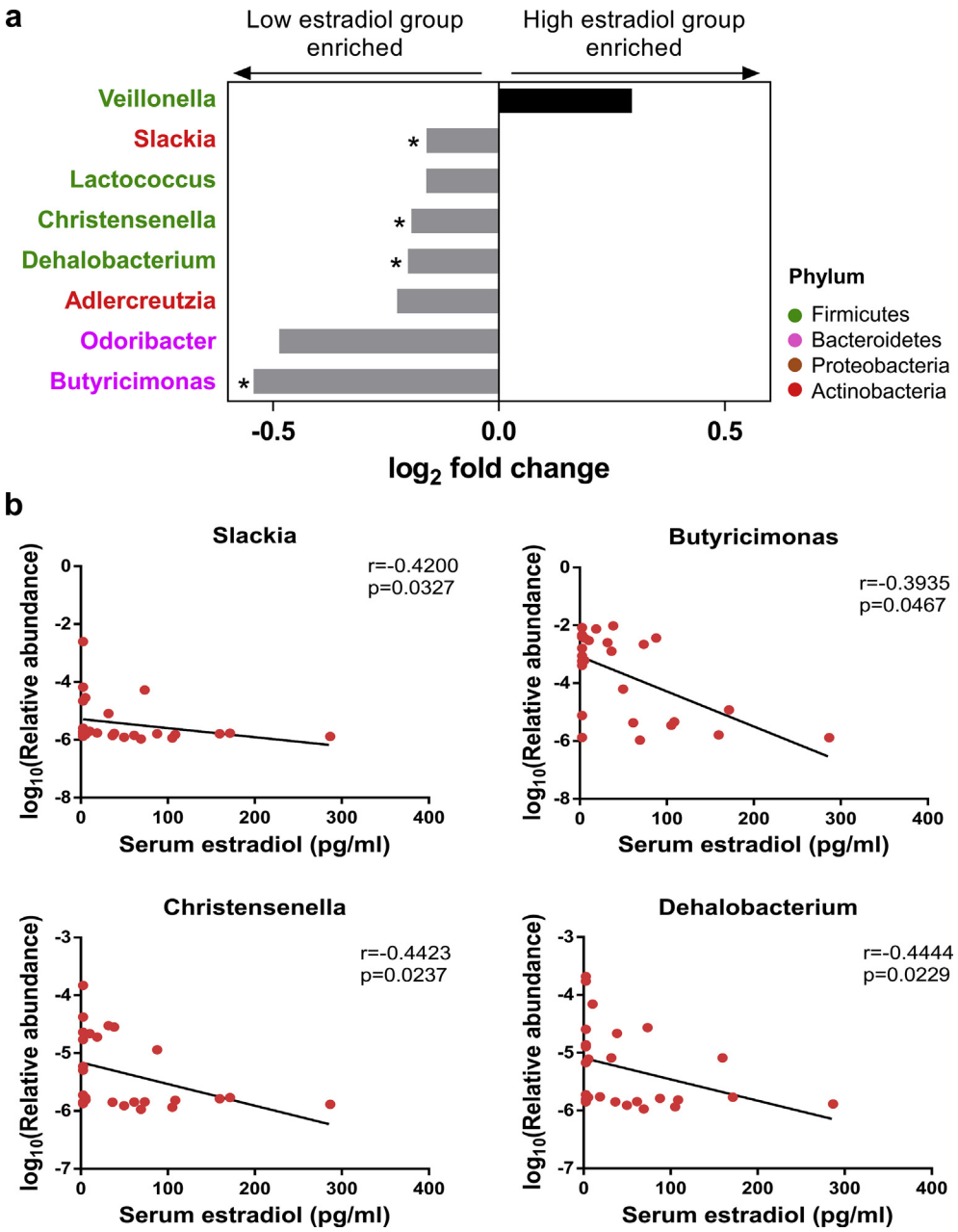

Fig. 5. Screening of gut microbial genera associated with serum estradiol concentration in women. **a** Eight most abundant genera that differ significantly between Low and High estradiol group in women as determined by the $\log_2$ of the fold change between two groups. High estradiol group with enriched taxa are indicated with a positive $\log_2$ fold change (black), and taxa enriched in the Low estradiol group have a negative value (grey). Asterisk represents statistical significance ($P < 0.1$; two-tailed Mann–Whitney U test). **b** Correlation between serum estradiol levels and relative abundance of four genera (*Slackia*, *Dehalobacterium*, *Christensenella*, and *Butyricimonas*) in women ($N = 26$). Linear regression is shown, and the Spearman correlation coefficient (r) and P-value are indicated.

estradiol levels might play some role in the women's physiology by changing gut microbial composition. Several mechanisms have been previously proposed to explain the influence of the gut microbiome on estrogen metabolism, termed the estrobolome [52]. Phytoestrogens and estrogen are metabolized by intestinal bacteria through bacterial secretion of β-glucuronidase and turned into bioavailable forms that are able to enter the bloodstream and act on estrogen receptors. However, we did not find that specific bacterial genera able to produce β-glucuronidase [17] were differentially enriched in the estradiol Low group compared to estradiol High group. Although we did not directly analyze β-glucuronidase enzymatic activity, our findings support the possibility that the distribution of estrogen-metabolizing gut microbial genera does not directly influence the serum estradiol concentrations of the host.

In contrast to women, structure of the fecal microbial community at the phylum level in the Low testosterone group of men remained similar to that found for the High testosterone group. This finding differs from a recent trial in mice where inducing hypogonadism was found to have increased the ratio of the *Firmicutes*/*Bacteroidetes* ratio among intestinal microbes [53]. Recent studies have also demonstrated associations between endogenous and exogenous testosterone and variations in the composition of the gut microbiota [53]. However, we have found that quantity of

several bacterial genera is significantly correlated with serum testosterone levels. There were significant positive correlations between the relative abundance of bacteria in the *Actinobacter*, *Dorea*, *Ruminococcus*, and *Megamonas* genera and serum testosterone concentrations. This was in accordance with several studies. For instance, a mouse model of polycystic ovary syndrome (PCOS) that showed a decrease of serum testosterone concentration was associated with increased *Ruminococcus* [54]. Likewise, *Ruminococcus* was reported to be significantly increase in neonatally-androgenized rats [55].

The current study has several limitations to consider. To begin, the BMI in our study sample were mostly within the overweight range, so that the data are not informative for persons within the lower range of BMI. Additionally, the study does not address social-environmental factors that might affect in shaping gut bacterial community. Another limitations are that the relatively small participants were recruited and menstrual cycle in women was not controlled. Although we found that specific serum testosterone or estradiol levels were associated with distinct features of the gut microbiome, a causal relationship cannot be verified in the present study. Furthermore, we examined the effect of testosterone in males not in female, therefore, a future research that determines the roles of testosterone played in communicating gut microbiota in females and vice versa, roles of estradiol in males will broaden our current understandings of the interactions in human.

To our knowledge, the present study is the first to report the relationship between intestinal bacterial community profiles and testosterone/estrogen status in humans. Gut microbiota signatures were analyzed in association with the predominant sex steroid hormone in men (testosterone) and women (estradiol), respectively. Men having high levels of testosterone and women with high levels of estradiol harbor more diverse profiles of bacterial community compared to their counterparts with low levels of the hormones. This suggests that sex hormones might contribute to maintaining gut health, as healthy subjects are known to have diverse gut flora. The set of intestinal bacteria that respond to the sex steroid hormone, testosterone, in men is distinguished from those in women, implying male and female sex hormones exert differential influences on the gut microbiome. This leads us to propose that there might be sex-dependent differences in the gut microbiome, although conflicting results have been reported in other human studies. Furthermore, our results could provide a foundation for further investigations of the sexual dimorphisms in gut microbiomes with respect to sex steroid hormone status.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.resmic.2019.03.003.

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