

Original Article

# Aging effects on the diurnal patterns of gut microbial composition in male and female mice

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**ABSTRACT** Composition of the gut microbiota changes with aging and plays an important role in age-associated disease such as metabolic syndrome, cancer, and neurodegeneration. The gut microbiota composition oscillates through the day, and the disruption of their diurnal rhythm results in gut dysbiosis leading to metabolic and immune dysfunctions. It is well documented that circadian rhythm changes with age in several biological functions such as sleep, body temperature, and hormone secretion. However, it is not defined whether the diurnal pattern of gut microbial composition is affected by aging. To evaluate aging effects on the diurnal pattern of the gut microbiome, we evaluated the taxa profiles of cecal contents obtained from young and aged mice of both sexes at daytime and nighttime points by 16S rRNA gene sequencing. At the phylum level, the ratio of Firmicutes to Bacteroidetes and the relative abundances of Verrucomicrobia and Cyanobacteria were increased in aged male mice at night compared with that of young male mice. Meanwhile, the relative abundances of Sutterellaceae, *Alloprevotella*, Lachnospiraceae UCG-001, and *Parasutterella* increased in aged female mice at night compared with that of young female mice. The Lachnospiraceae NK4A136 group relative abundance increased in aged mice of both sexes but at opposite time points. These results showed the changes in diurnal patterns of gut microbial composition with aging, which varied depending on the sex of the host. We suggest that disturbed diurnal patterns of the gut microbiome can be a factor for the underlying mechanism of age-associated gut dysbiosis.

## INTRODUCTION

The microbiota of the gut influences many aspects of host physiology and biological functions, such as mucosal integrity of the intestinal epithelial barrier, food digestion and absorption, and production of neurotransmitters and hormones [1]. The gut microbiota, which is dominated by bacteria, also plays an important role in a wide range of diseases beyond the intestine, such as metabolic syndrome and cancer [1]. For example, in the intestine of subjects with metabolic syndrome, the relative abundance of two dominant phyla, Firmicutes and Bacteroidetes, are changed

and bacterial diversity is reduced (gut dysbiosis). A causal role of gut microbiota in developing metabolic diseases has been further defined by gut microbiota transplantation, which replicates the phenotype of metabolic syndrome [2-4].

The gut microbiota also changes with aging. Specifically, the abundance of normally dominant bacteria decreases while opportunistic pathobionts increases and the diversity of the gut microbiota decreases in the gut of aged subjects [5]. These age-related changes in gut microbiota are implicated in developing low-grade chronic inflammation (inflammaging), which is a risk factor for morbidity and mortality in the elderly [6]. In addition,



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recent studies showed the association between age-related gut dysbiosis and the pathogenesis of atherosclerosis, type 2 diabetes, and neurodegeneration that are prevalent in the elderly [7-10]. Contributors to age-related changes in the gut microbiota include leaky gut, Western diet, changes in nutrient intake, medications, and increased hypothalamus-pituitary-adrenal axis activity [11,12]. However, a causal relationship and the underlying mechanism of age-related gut dysbiosis are not still clearly defined.

Circadian rhythms are present in most organisms and synchronize important physiological activities such as sleep/wake, hormone release, and metabolism [13]. A master clock in the suprachiasmatic nucleus of the hypothalamus regulates rhythmicity of physiology, and the environmental light/dark cycle serves as the strongest signal to synchronize circadian rhythms [14]. It has been reported that the abundance of certain gut bacteria also oscillates rhythmically during the course of a day [15-17]. For example, the proportion of the phylum Firmicutes in the gut peaks during dark phase (active phase) and bottoms during light phase (rest phase). Meanwhile, the proportions of the phyla Bacteroidetes and Verrucomicrobia exhibit antiphasic patterns. The diurnal rhythm of gut microbiota is disrupted by molecular clock gene deficiency, high fat diet, food restriction, and shifts in the light/dark cycle, which influence the host circadian physiology and the susceptibility to metabolic diseases [15-19]. These studies indicate that microbial rhythm is interrelated to the host circadian rhythm. In general, as the circadian rhythm declines with aging [20,21], it can be assumed that the diurnal composition of the gut microbiota may also change with aging. However, their diurnal patterns in aged subjects compared with those of young subjects has not been determined.

We previously reported that 20-month-old mice of both sexes are reproductively senescent and exhibit increased visceral adiposity, a feature of aging, compared with young 10–12 weeks-old mice [22,23]. In this study, to evaluate the effects of aging on the diurnal patterns of the gut microbiota composition, we compared the composition of gut microbiota in the cecum of young and aged male and female young and aged mice sampled at two different time points of light/dark periods.

## METHODS

### Animals and sample collection

Male and female C57BL/6 mice (6 weeks old) were purchased from Orient Bio Inc. (Seongnam, Korea) and maintained in a specific pathogen-free animal facility at Ewha Womans University Medical College on a 12-h light/dark cycle (8:00 AM, light on; 8:00 PM, light off) at  $22 \pm 2^\circ\text{C}$ . It has been reported that mice fed normal chow ad libitum exhibit cyclical fluctuations in phyla within the gut microbiome [15]. Therefore, in the present study, mice were given standard rodent chow, and tap water ad libitum.

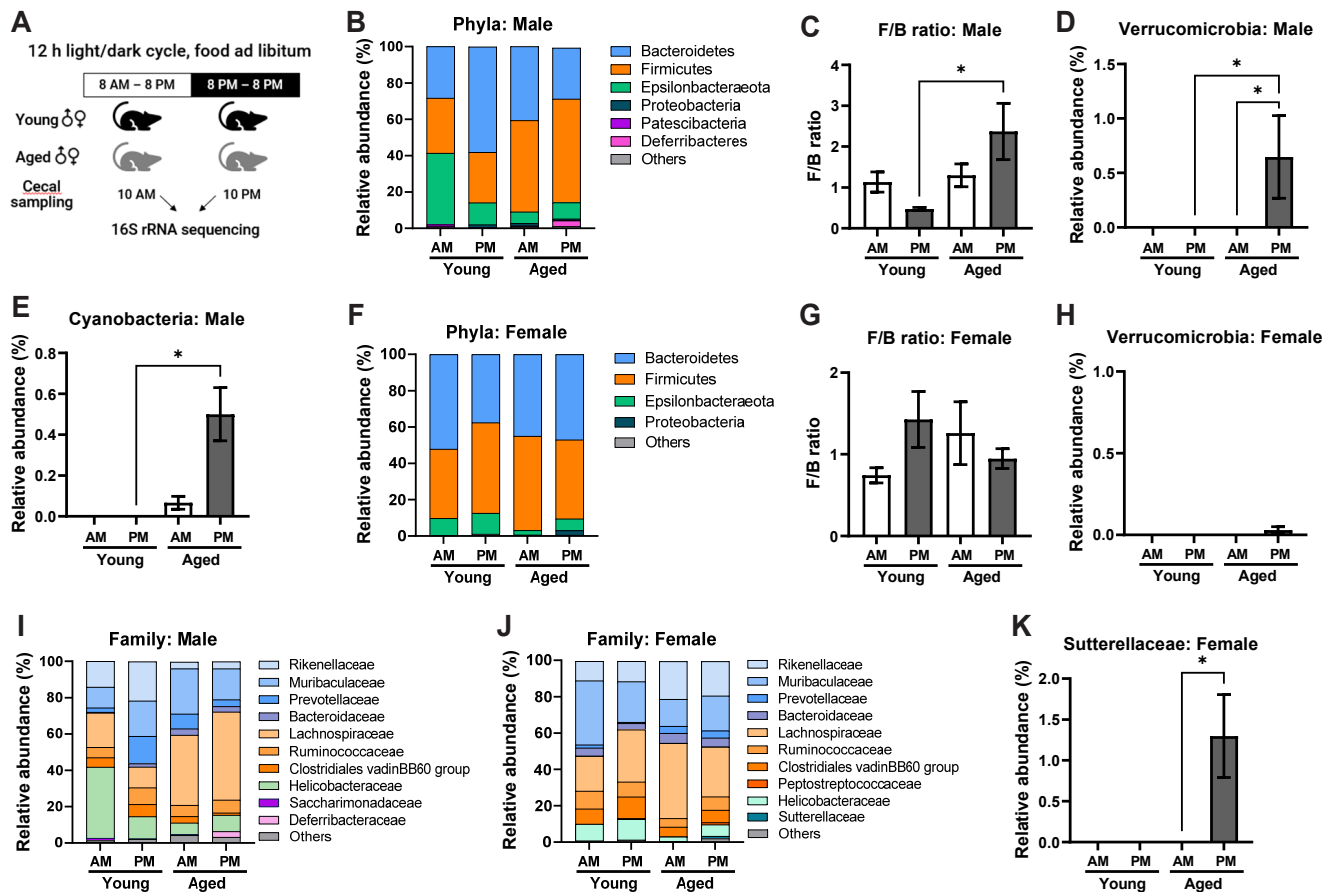
Experiments were performed using young adult mice (2.5 months old), and aged mice (20 months old). Three mice from different cages were used for gut microbiota analysis, and therefore, six young (body weights of male and female were  $25.50 \pm 0.48$  g and  $21.17 \pm 0.48$  g, respectively) and six aged (body weights of male and female mice were  $38.37 \pm 1.53$  g and  $28.67 \pm 0.71$  g, respectively) mice of each sex were used ( $n = 3$  per sampling time point). The cecum of the mice was collected under terminal sodium pentobarbital anesthesia (120 mg/kg, intraperitoneal) at 10 AM for the light period of day (AM) and 10 PM for the dark period of night (PM, Fig. 1A). For the PM sampling, the mice were anesthetized in the dark and the cecum then obtained under light. The cecum specimens were stored at  $-80^\circ\text{C}$  until analyses. All procedures were approved by the Institutional Animal Care and Use Committee at the Medical School of Ewha Womans University (EWAH MEDICACUC past-057) and conformed to the international guidelines for the ethical use of experimental animals.

### Bacterial DNA extraction and 16S ribosomal RNA gene sequencing

DNA of the cecal contents was extracted using a DNA Fast Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The 16S ribosomal RNA (rRNA) gene analysis was performed at Ebiogen Inc. (Seoul, Korea), as previously described [24]. Briefly, each sequenced sample was prepared according to the Illumina 16S Metagenomic Sequencing Library protocols and the genes were amplified using 16S V3-V4 primers. The final products were normalized, pooled, and the size of the libraries verified using the Agilent TapeStation DNA ScreenTape D1000 system (Agilent Technologies, Santa Clara, CA, USA). Finally, the pooled libraries were sequenced ( $2 \times 300$ ) using the MiSeq platform (Illumina, San Diego, CA, USA). The amplicon error was modelled from paired fastq using DADA2 (ver.1.10.1) and filtered out noise sequence, corrected errors in marginal sequences, removed chimeric sequences and singleton, and then dereplicated those sequences. Each taxon level was classified based on the SILVA rRNA (region V3-V4) reference database (<https://www.arb-silva.de/>) using a q2-feature classifier. Each diversity metric was calculated by a q2-diversity program. The percent of total reads was calculated for each mouse and then averaged per sampling time point.

### Statistical analysis

Data are expressed as the mean  $\pm$  standard error of the mean (SEM). Differences between groups were analyzed using Kruskal-Wallis followed by the Dunn's test. Prism9.0 software (GraphPad Software, Inc., La Jolla, CA, USA) was used to perform the statistical analyses. Statistical significance was set at  $p < 0.05$ .



**Fig. 1. Diurnal differences of the gut microbiota between young and aged mice at the phylum and family levels.** (A) Experimental scheme of gut sampling times in young and aged mice according to light/dark cycle. (B–E) Mean gut microbiota values of cecum collected at 10 AM (AM) and 10 PM (PM) samples of young and aged male mice at the phylum level. (B) Stacked bar chart of microbiota composition of phyla present at > 1% relative abundance of male mice. (C) Firmicutes to Bacteroidetes (F/B) ratio of male mice. (D) Relative abundance of Verrucomicrobia of male mice. (E) Relative abundance of Cyanobacteria of male mice. (F) Stacked bar chart of microbiota composition of phyla present at > 1% relative abundance of females. (G) F/B ratio of female mice. (H) Relative abundance of Verrucomicrobia of female mice. (I–K) Mean gut microbiota values of AM and PM cecum samples of young and aged mice at the family level. (I) Stacked bar chart of microbiota composition of families present at > 1% relative abundance of male mice. (J) Stacked bar chart of microbiota composition of families present at > 1% relative abundance of female mice. (K) Relative abundance of Sutterellaceae of female mice.  $n = 3/\text{group}$ ,  $*p < 0.05$ .

## RESULTS

### Diurnal changes of gut microbiota composition with aging at the phylum and family levels

The number of operational taxonomic units (OTUs) and diversity, including alpha-diversity (intrasample diversity), beta-diversity (intersample diversity), and evenness, were compared between AM and PM samples of young and aged mice to determine diurnal patterns in general characteristics of gut microbiota with aging. We found no statistical differences between the AM and PM samples in either young or aged mice of either sex for any of either sex for any of the parameters investigated (Table 1). Also, no statistical changes were observed between young and aged mice at neither AM nor PM, although the number of OTUs in both AM and PM decreased in aged mice compared to those

of young mice. These results are consistent with a previous study that reported decreased bacterial richness and diversity without statistical significance in cecal samples from aged mice compared to those of young mice [25].

At the phylum level, there were also no changes with aging of the diurnal patterns of bacteria with more than 1% relative abundance, regardless of sex (Table 2, Fig. 1B, F). However, the ratio of Firmicutes to Bacteroidetes (F/B ratio), which is indicative of dysbiosis [26], was significantly increased in aged male mice compared with that of young male mice for the PM samples ( $p = 0.04$ ; Fig. 1C). The relative abundance of the minor phyla Verrucomicrobia and Cyanobacteria were also increased in aged male mice compared with that of young male mice for the PM samples (both  $p = 0.04$ ; Fig. 1D, E). Meanwhile, there were no diurnal changes in F/B ratios between young and aged female mice (Fig. 1G). In addition, the relative abundance of Verrucomicrobia was slightly in-

**Table 1. Characteristics of metagenome sequencing data and diversity of microbiome from cecal contents of male and female mice**

Characteristics	Young		Aged		p-value
	AM	PM	AM	PM	
Male					
No. of OTUs	317.0 ± 10.97	363.3 ± 17.89	289.7 ± 16.37	320.3 ± 39.07	0.30
Alpha diversity (Shannon Index)	4.83 ± 0.26	5.38 ± 0.10	6.30 ± 0.11	4.91 ± 0.73	0.16
Beta diversity	0.51 ± 0.03	0.61 ± 0.04	0.52 ± 0.06	0.57 ± 0.05	0.36
Faith's phylogenetic diversity	18.08 ± 2.49	23.51 ± 2.63	18.71 ± 2.59	23.51 ± 6.49	0.67
Evenness (Peilou Index)	0.58 ± 0.03	0.63 ± 0.01	0.77 ± 0.01	0.59 ± 0.07	0.05
Female					
No. of OTUs	387.3 ± 30.37	398.7 ± 17.61	252.0 ± 10.00	361.7 ± 11.20	0.08
Alpha diversity (Shannon Index)	6.17 ± 0.28	6.05 ± 0.06	4.43 ± 0.04	5.95 ± 0.16	0.07
Beta diversity	0.43 ± 0.00	0.40 ± 0.01	0.31 ± 0.01	0.48 ± 0.08	0.10
Faith's phylogenetic diversity	19.76 ± 1.16	19.89 ± 0.45	15.85 ± 0.43	23.88 ± 3.98	0.07
Evenness (Peilou Index)	0.72 ± 0.024	0.70 ± 0.00	0.56 ± 0.01	0.70 ± 0.02	0.06

Values are presented as mean ± SEM. OTUs, operational taxonomic units.

**Table 2. The diurnal relative abundance of gut microbiota at the phylum level in young and aged mice**

Phylum	Young		Aged		p-value
	AM	PM	AM	PM	
Male					
Bacteroidetes	28.47 ± 5.33	58.10 ± 1.70	40.67 ± 4.61	28.27 ± 7.15	0.08
Firmicutes	30.17 ± 5.83	27.70 ± 2.70	50.30 ± 4.53	57.27 ± 3.98	0.08
Epsilonbacteraeota	39.17 ± 8.21	12.13 ± 3.28	6.30 ± 1.86	8.97 ± 2.97	0.08
Proteobacteria	0.16 ± 0.09	1.57 ± 0.92	1.30 ± 0.32	0.90 ± 0.10	0.05
Patescibacteria	1.24 ± 1.14	0.30 ± 0.06	0.50 ± 0.06	0.03 ± 0.03	0.09
Deferribacteres	0.01 ± 0.01	0.01 ± 0.01	0.11 ± 0.09	3.08 ± 1.91	0.23
Female					
Bacteroidetes	52.13 ± 3.77	37.54 ± 5.49	44.96 ± 6.23	47.01 ± 4.26	0.32
Firmicutes	38.09 ± 2.59	49.84 ± 5.79	51.85 ± 7.18	43.50 ± 2.52	0.21
Epsilonbacteraeota	9.36 ± 2.53	11.47 ± 0.68	2.50 ± 1.24	6.37 ± 3.47	0.13
Proteobacteria	0.04 ± 0.01	0.35 ± 0.51	0.59 ± 1.15	2.52 ± 0.56	0.33

Values are presented as mean ± SEM. Phyla listed at > 1% relative abundance.

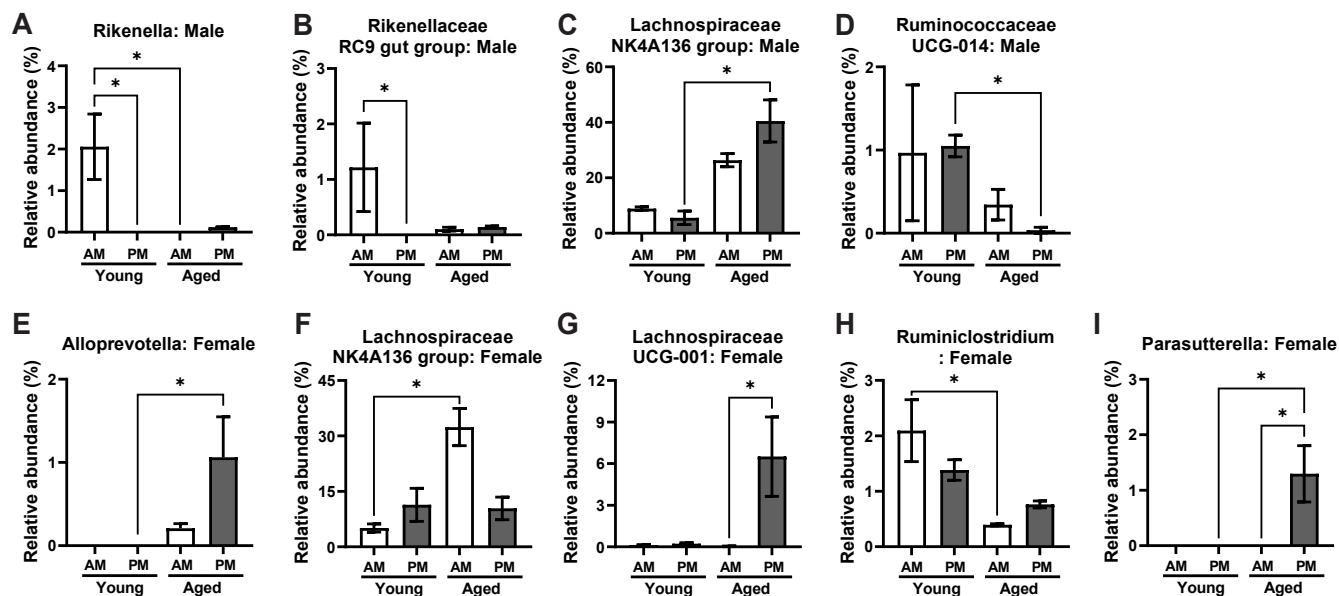
creased in aged female mice at PM, but no statistical significance was observed between young and aged female mice (Fig. 1H). Unlike the results of male mice, Cyanobacteria were not detected in either young or aged female mice.

At the family level, differences in diurnal patterns of bacteria with more than 1% relative abundance were not observed between young and aged mice in either sex (Table 3, Fig. 1I, J), except for the family Sutterellaceae that was not observed in male mice. The relative abundance of Sutterellaceae increased in aged female mice compared with that in young female mice at PM ( $p = 0.04$ ; Fig. 1K). These findings indicate that the diurnal patterns of some components of the gut microbiome at phylum and family levels change with aging and there are sex-associated dissimilarities.

### Diurnal changes of gut microbiota composition with aging at the genus level

The gut microbiome changes at genus level with aging [5,25], and therefore we determined diurnal patterns of bacteria genera present at > 1% abundance.

In male mice, four bacteria showed differences in their diurnal patterns. In young male mice, the relative abundances of *Rikenella* and *Rikenellaceae RC9 gut group* of the phylum Bacteroidetes were significantly increased in AM samples compared with those in PM ( $p = 0.04$  and  $p = 0.01$ , respectively); however, their relative abundances were decreased in aged male mice at both time points (Fig. 2A, B). The significant decrease in the relative abundance of *Rikenella* in aged male mice compared with that in young male mice was especially observed in AM samples ( $p = 0.04$ ; Fig. 2A). The relative abundance of *Lachnospiraceae NK4A136 group* of the phylum Firmicutes was markedly increased in aged male mice compared with that in young male mice and the increase



**Fig. 2. Diurnal differences of gut microbiota between young and aged mice at the genus level.** (A–D) Mean gut microbiota values of cecum collected at 10 AM (AM) and 10 PM (PM) samples of young and aged male mice. (A) Relative abundance of *Rikenella*. (B) Relative abundance of *Rikenellaceae RC9 gut group*. (C) Relative abundance of *Lachnospiraceae NK4A136 group*. (D) Relative abundance of *Ruminococcaceae UCG-014*. (E–I) Mean gut microbiota values of AM and PM cecum samples of young and aged female mice. (E) Relative abundance of *Alloprevotella*. (F) Relative abundance of *Lachnospiraceae NK4A136 group*. (G) Relative abundance of *Lachnospiraceae UCG-001*. (H) Relative abundance of *Ruminiclostridium*. (I) Relative abundance of *Parasutterella*.  $n = 3/\text{group}$ , \* $p < 0.05$ .

**Table 3. The diurnal relative abundance of gut microbiota at the family level in young and aged mice**

Family	Young		Aged		p-value
	AM	PM	AM	PM	
<b>Male</b>					
Rikenellaceae	14.20 ± 6.35	21.57 ± 5.64	3.60 ± 0.86	3.93 ± 0.45	0.11
Muribaculaceae	11.37 ± 3.50	19.47 ± 2.86	24.83 ± 2.28	17.00 ± 4.54	0.12
Prevotellaceae	2.50 ± 2.11	15.07 ± 1.30	8.17 ± 2.86	3.77 ± 2.13	0.06
Bacteroidaceae	0.40 ± 0.12	1.97 ± 0.37	3.53 ± 0.95	3.07 ± 0.95	0.05
Lachnospiraceae	18.87 ± 4.78	11.30 ± 3.38	38.58 ± 4.10	48.45 ± 6.13	0.08
Ruminococcaceae	5.69 ± 1.62	9.20 ± 0.99	6.17 ± 1.64	7.04 ± 2.01	0.61
Clostridiales vadin BB60 group	5.22 ± 2.01	6.72 ± 0.79	3.50 ± 1.42	1.27 ± 0.29	0.24
Helicobacteraceae	39.16 ± 8.22	12.13 ± 3.28	6.29 ± 1.87	8.97 ± 2.99	0.08
Saccharimonadaceae	1.24 ± 1.13	0.32 ± 0.06	0.50 ± 0.04	0.05 ± 0.05	0.06
Deferribacteraceae	0.01 ± 0.01	0.01 ± 0.01	0.11 ± 0.09	3.08 ± 1.91	0.23
<b>Female</b>					
Rikenellaceae	10.63 ± 6.78	11.16 ± 1.91	20.85 ± 3.48	18.94 ± 1.95	0.24
Muribaculaceae	35.37 ± 2.84	22.29 ± 1.88	14.78 ± 4.71	19.19 ± 5.43	0.08
Prevotellaceae	1.61 ± 0.06	0.64 ± 0.33	3.89 ± 1.12	3.94 ± 1.72	0.05
Bacteroidaceae	4.47 ± 0.26	3.36 ± 2.39	5.43 ± 1.11	4.90 ± 0.88	0.86
Lachnospiraceae	19.10 ± 3.56	28.76 ± 4.39	41.38 ± 7.42	27.34 ± 2.48	0.05
Ruminococcaceae	9.85 ± 1.67	8.18 ± 0.56	4.70 ± 0.37	7.37 ± 0.72	0.06
Clostridiales vadin BB60 group	8.34 ± 1.14	11.90 ± 2.03	5.40 ± 1.081	6.90 ± 0.66	0.09
Peptostreptococcaceae	0	0.43 ± 0.15	0.01 ± 0.01	1.13 ± 0.49	0.15
Helicobacteraceae	9.36 ± 2.53	11.47 ± 0.68	2.50 ± 1.24	6.37 ± 3.47	0.13
Sutterellaceae	0	0	0	1.30 ± 0.51	0.04

Values are presented as mean ± SEM. Families listed at > 1% relative abundance.

was significant in PM samples ( $p = 0.04$ ; Fig. 2C). In contrast, the relative abundance of *Ruminococcaceae UCG-014*, which also belongs to the phylum Firmicutes, was decreased in aged male

mice compared with that in young male mice, especially in PM samples ( $p = 0.04$ ; Fig. 2D).

In female mice, there were changes in the diurnal patterns

of five bacteria, which differed from those of male mice. *Alloprevotella* and *Parasutterella* were not detected in young female mice, but were present at significant levels in aged female mice, especially in PM samples (both  $p = 0.04$  vs. young female mice; Fig. 2E, I). The increased level of *Parasutterella* may have resulted from the increased relative abundance of the family Sutterellaceae (Fig. 1I). The relative abundance of *Lachnospiraceae* NK4A136 group was also significantly increased in aged female mice compared with that in young female mice in AM samples ( $p = 0.02$ ; Fig. 2F), which was different from that observed in the aged male mice. The increased relative abundance of *Lachnospiraceae* UCG-001 was statistically significant in PM samples compared with AM samples of aged female mice ( $p = 0.03$ ; Fig. 2G). Meanwhile, the relative abundance of *Ruminiclostridium*, which also belongs to the phylum Firmicutes, decreased in aged female mice compared with young female mice, especially in AM samples ( $p = 0.03$ ; Fig. 2H). Taken together, these results indicate that the diurnal patterns of gut microbiota composition are affected at the genus level by aging with sex-associated dissimilarities.

## DISCUSSION

The present study showed that the relative abundance of certain gut bacteria at several taxa levels changed with age between day and night times. In addition, the diurnal changes with aging in the gut microbiota composition were dissimilar between sexes. To the best of our knowledge, this is the first study to demonstrate diurnal changes in the relative abundance of some bacteria in the gut of aged mice of both sexes compared to young mice.

It has been reported that age-associated increase of F/B ratio can be resulted from increased Firmicutes and/or decreased of Bacteroidetes abundances [25-28]. Interestingly, we observed that the significant increase in the F/B ratio of aged male mice was only at nighttime. The increased F/B ratio may have resulted from increased Firmicutes abundance, which was also reflected by the increased abundance of *Lachnospiraceae* NK4A136 group, which belongs to the phylum Firmicutes, at nighttime. Previous studies showed that the increase of F/B ratio was linked to increases of lipopolysaccharide levels in the plasma and feces of aged male mice [25], and increased abundance of Cyanobacteria in mice with high F/B ratio can be related to the severity of aging-associated dry eye [29]. We also observed the increases in F/B ratio and the relative abundance of Cyanobacteria in aged male mice at nighttime. Additionally, the relative abundance of Verrucomicrobia, which was increased in aged male mice at nighttime in the present study, has been positively correlated with plasma concentration of inflammatory cytokine interferon gamma in patients with Parkinson's disease [30]. Contrary to the results of male mice, neither the F/B ratio nor microbiota composition at the phylum level showed any differences between young and aged female mice in the present study, which were comparable to a previous

study reporting no changes in F/B ratio of fecal samples collected from three different age groups (young, middle-aged, and old) [31]. Based on these previous studies, we suggest that altered diurnal patterns of F/B ratio and abundance of Verrucomicrobia and Cyanobacteria have roles in inflammaging and severity of age-associated diseases, at least in males.

At the family level, only the nighttime Sutterellaceae abundance differed significantly between young and aged female mice. The increase of Sutterellaceae abundance was again reflected by the increase of *Parasutterella* genus abundance in aged female mice at nighttime. A study reported that the abundance of Sutterellaceae increased in the feces of patients with irritable bowel syndrome [32], but the roles of Sutterellaceae and *Parasutterella* in aging and sex differences are remained to be determined. At genus level, more bacteria showed significant changes in diurnal patterns of their relative abundance in the gut of aged mice of both sexes, differentially. *Lachnospiraceae* NK4A136 group was dominant and their relative abundance markedly increased in the gut of aged mice of both sexes; however, the time points for the increase in their abundance were opposite in aged male (nighttime) and female (daytime) mice. It has been demonstrated the increase in *Lachnospiraceae* NK4A136 group abundance in mouse models of colitis and diet-induced obesity, suggesting pathological roles for these bacteria in diseases-associated inflammation [33,34]. The other genera such as *Rikenella*, *Ruminococcaceae* UCG-014, *Alloprevotella*, and *Parasutterella*, that showed changes in diurnal pattern of their abundance in the present study, have been also suggested to be involved in several diseases. *Rikenella* abundance decreased in the mouse gut of an Alzheimer's disease (AD) model, and gut-flora targeted photobiomodulation therapy reversed the changes in gut microbiome with improved cognitive function and brain pathologies [35]. The authors suggest that interventions targeting gut flora can be a treatment modality for AD [35]. Butyrate-producing *Ruminococcaceae* UCG-014 abundance also decreased in the mouse gut of a ulcerative colitis carcinogenesis model [36]. Meanwhile, the increases in the abundances of *Alloprevotella* and *Parasutterella*, which were also increased in aged female mice at nighttime in the present study, were observed in patients with irritable bowel syndrome [37,38], and *Alloprevotella* was one of the pathogens associated with chemotherapy-induced diarrhea in mice [39]. Furthermore, a recent study reported that ovariectomy, a model of menopause, induced osteoporosis and changed abundances of *Alloprevotella* and *Parasutterella* in the gut of female mice compared to the sham control, suggesting their roles in regulating bone homeostasis in postmenopausal women [40]. Considering that these taxa involved in the diseases with inflammation or aging, and that age-associated gut dysbiosis contributes to inflammaging [6], it is possible that diurnal changes in the abundance of certain gut bacteria may make the host vulnerable to diseases in which onset and symptoms worsen depending on the day or night. Typically, it is well known that osteoarthritis and Parkinson's disease symptoms are worse in the evening and

night, and acute myocardial infarct incidents are most frequent in the morning [41]. However, the implications of aging-associated diurnal changes in the gut microbiota with respect to host health should be determined in further studies.

Sex differences in gut microbial composition, abundance, and diversity have been previously demonstrated, and both mice and human studies have shown the major roles of sex hormones in these difference [42-44]. Importantly, it has been suggested that sex-specific gut microbiome composition contributes to the development of sex-biased autoimmune diseases, cardiovascular diseases (CVD) and AD [42,45,46]. For example, the development of autoimmunity, prevalent in females, was reduced in nonobese diabetic female mice by gut microbiota transfer from adult male mice via the increase of testosterone level [42]. Sex differences in the prevalence of CVD and AD have been related with the differences in branched-chain amino acids and short-chain fatty acids derived from the gut microbiome [45,46]. Regarding a diurnal pattern of gut microbiota between sexes, only one study analyzed fecal pellets of young adult mice sampled every 4 h for 48 h [16]. The authors found that gut microbial rhythmicity in the composition during a day was more pronounced in young adult female mice than in male mice, and that the clock *Bmal1* gene deletion altered the fecal microbial composition in a sex-dependent manner [16]. Although we did not directly compare the relative abundances of taxa between sexes, our results showed that diurnal patterns of gut microbiota composition differed according to the sex, of not only young but also aged mice. The rhythmicity and taxa in differences shown in the present study were not the same as those reported in the previous studies. These discrepancies may have resulted from differences in the experimental methods, such as sampling times (one-time sampling per light/dark period), intervals (sampling at 2 h after light turn on/off), and sites (cecum versus feces), as well as the small study size, which were also limitations of our study. As the focus of the present study was the aging effects on the diurnal pattern of gut microbiota, rather than sex differences in the gut microbiome, further studies to determine taxa differences during a day between sexes in aged subjects and their implications in host health are required.

In conclusion, we found that the relative abundance of some taxa at the phylum, family, and genus levels were changed according to light and dark periods in aged mice compared with those of young mice for both sexes. In addition, diurnal patterns and their changes in the gut microbiota with aging were dissimilar for males and females. We suggest that changes in the diurnal pattern of the gut microbiota composition contribute to age-associated gut dysbiosis with differences occurring between the sexes.

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## CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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