Gender Difference and Dietary Supplemental Vitamin B₆: Impact on Colon Luminal Environment

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(Received October 29, 2017)

Summary Colon diseases can be affected by several factors such as gender difference and dietary supplemental vitamin B₆ (B₆). The nutritional status of B₆ is affected by gender difference, leading us to hypothesize that gender difference affects colon luminal environment, which is dependent on B₆ status. To investigate this hypothesis, we fed male and female rats a diet containing 1 mg, 7 mg, or 35 mg pyridoxine HCl/kg diet for 6 wk. We found significantly higher fecal mucin levels in female rats compared to those in male rats. Supplemental B₆ significantly increased fecal mucins and was particularly profound in the female rats. The abundances of cecal and fecal Akkermansia muciniphila (mucin degrader) were unaffected. The fecal mucin levels were significantly correlated with colonic free threonine and serine and with gene expression of colon MUC16, implying that the combined effect of gender and dietary B₆ on fecal mucins was mediated by the alteration in the levels of such amino acids and MUC16 expression. This study further showed the significant effects of gender difference on colonic free amino acids such as threonine, ornithine, asparagine/aspartate ratio, and glutamine/glutamate ratio, cecal and fecal Lactobacillus spp. levels, and colonic gene expressions of MUC16 and TLR8, the factors relating to colon health and diseases. Therefore, our findings suggest that gender difference and dietary B₆ may have an impact on colon diseases by modulating these parameters.

Key Words gender difference, vitamin B₆, colon luminal environment, mucins, microflora

Many diseases, such as brain diseases, autoimmune diseases, and colon diseases, are differently expressed in men and women. Alzheimer disease (AD) and Parkinson disease (PD) are brain diseases that are influenced by gender difference. For example, females are affected by AD progress more often to severe cognitive dysfunction (1). A significantly higher incidence rate of PD was found among men with the relative risk being 1.5 times greater in men than women (2). Autoimmune diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) also emerge in consequence of gender difference. For example, the disease progression in female patients with RA were more worse compared with men (3). SLE tends to run a more severe course in males than in females, with a worse prognosis (4). Similarly, colon diseases such as intestinal bowel syndrome (IBS), Crohn’s disease (CD), ulcerative colitis (UC), and colon cancer are affected by gender difference. For example, compared to men, women are more likely to get IBS (5), girls appear to have an overall more severe course of pediatric CD (6), and women experience more constipation symptoms and have abnormal bowel habits more frequently than men do (7). However, CD in children <10 y affects predominantly boys (8), and the effects of UC on the incidence of colorectal cancer are greater in male patients than those in female patients (9).

The suggested mechanisms responsible for the effects of gender on the diseases include biological differences such as hormones and chromosomes (10, 11) as well as environmental factors such as nutrition (12) and microbiota (13, 14). Inflammation (15) and metabolisms (16, 17) are also responsible for the influence of gender on diseases. Higher inflammation in women is considered to be associated with higher incidence of diseases (15). Previous studies have reported the possible mechanisms of the gender effects on colon diseases. Steegenga et al. (18) reported that molecular sexually dimorphic effects between males and females in the small intestine and colon of prepubescent mice determined differences in physiological functioning and in disease predisposition. Sex hormones had a crucial role in the regulatory
mechanisms of the brain-gut-microbiota axis involved in the pathophysiology of IBS (19). Furthermore, Meleine and Matricon (20) reported that ovarian hormones modulated IBS and estrogen receptors were found to have implications in colorectal carcinogenesis (21). Meanwhile, Ams-Langgraf et al. (22) reported that testosterone promoted early adenomagenesis and the enhanced susceptibility of males to colonic adenomas. However, there is limited information on the role of gender difference in the colon luminal environment. An understanding of the colon luminal environment is very important because changes in mucins (23), immunoglobulin A (IgA) (24), and microflora (25) can be a sign of the onset of colon diseases. Mucins have an important role in gastrointestinal barrier function (23). IgA is one of the most important effector molecules in the gastrointestinal immune system because it is the first line of defense in protecting the intestinal epithelium from enteric toxins and pathogenic microorganisms (24).

Vitamin $B_6$ ($B_6$) is an essential water-soluble vitamin required for normal growth and development in mammals (26). Pyridoxal 5′-phosphate (PLP), the biologically active form of $B_6$, acts as a co-factor in over 140 distinct enzyme reactions that are involved in the metabolisms of proteins, lipids, and carbohydrates, neurotransmitters, nucleic acids, one-carbon units, immune modulatory metabolites and others (27). Beyond its role as a co-factor, $B_6$ has preventive roles in certain diseases including colon diseases, such as colitis, intestinal bowel disease (IBD), and colon cancer. Possible mechanisms by which $B_6$ prevents colon diseases have been reported. Suplemental $B_6$ was found to protect colon DNA against damage in female rats with colitis (28). Dietary $B_6$ intake was shown to modulate colonic inflammation in the IL10 knockout murine model of IBD (29). Our previous studies have suggested that $B_6$ exhibits an anti-tumor effect by reducing cell proliferation, oxidative stress, inflammation, and angiogenesis (30–33). Additionally, we reported an increase of fecal mucins in rats from dietary supplemental $B_6$ (34). However, there is still limited information about the effect of dietary $B_6$ on the colon luminal environment.

As mentioned above, there is growing evidence that the incidence of colon diseases is affected by gender difference. Additionally, accumulating studies have suggested the preventive role of dietary $B_6$ on colon diseases and the status of $B_6$ is affected by gender difference (35). Thus, in this study, we hypothesized that gender difference modulates the colon luminal environment, which is dependent upon $B_6$ status. To examine this hypothesis, this study investigated the effects of gender difference on the colon luminal environment in male and female rats fed diets containing different levels of $B_6$.

**MATERIALS AND METHODS**

**Animals and diets.** A total of 24 male and 24 female Sprague-Dawley rats (3 wk old) were purchased from the Hiroshima Laboratory Animal Center (Hiroshima, Japan) and were maintained according to the “Guide for the Care and Use of Laboratory Animals” established by Hiroshima University. This study was approved by the Ethics Committee of Hiroshima University (approval No. C15-12). The rats were housed individually in an air-conditioned room at 23–24°C under a 12-h light/dark cycle (lights on from 08:00–20:00). Following acclimatization with a non-purified commercial rodent diet (MF, Oriental Yeast Co., Ltd., Tokyo) for 7 d, the male rats (mean body weight, 113 g) or female rats (mean body weight, 109 g) were randomly assigned to one of three groups ($n=8$ rats per group). The rats were provided free access to experimental diets and drinking water for 6 wk. The basal diet (36) comprised the following components (g/kg diet): α-cornstarch, 302; casein, 200; sucrose, 200; corn oil, 200; cellulose powder, 50; AIN-93G mineral mixture (37), 35; AIN-93 vitamin mixture (PN free) (37), 10; and l-cystine, 3. Pyridoxine (PN) HCl (Nacalai Tesque, Inc., Kyoto, Japan) was supplemented to the basal diet at 1, 7, or 35 mg PN HCl per kg of diet. The level of PN HCl diet recommended in the AIN-93 diet is 7 mg/kg PN HCl (37), while 1 mg/kg PN HCl diet is reported to be the minimum level required to prevent growth depression caused by $B_6$ deficiency (38). Feces were collected for the final 2 d. At the end of the feeding period, the rats were euthanized by decapitation following anesthesia (13:00–15:00) with inhalation exposure of isoflurane (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in the desiccator to reduce the suffering. Blood was collected, and serum was separated by centrifugation at 2,000 $\times g$ for 20 min and stored at $-80^\circ$C. The cecum was immediately excised, and its contents were completely removed, weighed, and stored at $-80^\circ$C until subsequent analysis of microflora.

**Serum PLP analysis.** The analysis of serum PLP was conducted using high-performance liquid chromatography (HPLC) (39). Briefly, $B_6$ from serum samples was extracted with 3 N perchloric acid (PCA), and PLP was converted to pyridoxic acid 5′-phosphate and measured using HPLC with a fluorometric detector.

**Colon free amino acids analysis.** The analysis of colon free amino acids was done by an amino acid analyzer (JLC-500; JEOL, Tokyo, Japan). To measure the concentrations of free amino acids, we homogenized the colon on ice with 3% sodium salicyclic acid. After centrifugation at 2,000 $\times g$ at 4°C for 20 min, the supernatant was collected, filtered through a 0.22-μm-pore-size membrane filter, and immediately stored at $-80^\circ$C until analysis. Quantification of free amino acids was carried out by an amino acid analyzer (JLC-500; JEOL). Amino acid mixture standard solutions (type AN-2 and type B) were used as standard solutions (Wako).

**Cecal and fecal microflora analysis.** The analysis of bacterial genomic DNA was done by real-time quantitative polymerase chain reaction (qPCR) using StepOneTM Real-Time PCR System (Applied Biosytems, Foster City, CA). Bacterial genomic DNA was isolated from the cecum and feces using the UltraClean Fecal DNA extraction kit (Mo Bio Laboratories, Carlsbad, CA) according to the manufacturer’s instructions. Bacterial groups (Table 1) were quantified by real-time qPCR using the...
StepOne Real-Time PCR System (Applied Biosystems). Real-time qPCR was performed in a reaction volume of 20 μL containing 10 μL SYBR qPCR mix (Toyobo, Osaka, Japan), 200 nmol/L each of the forward and reverse primers (40–44), and 2 μL cecal or fecal DNA samples. The reaction conditions were 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, 55°C for 15 s, and 72°C for 30 s. The fluorescent products were detected at the last step of each cycle. Melting curve analysis was performed after amplification to distinguish the tar-
**Table 2. Effects of gender difference and dietary B6 on body weight, food intake and serum PLP in rats.**

| Parameters            | Male   | Female | Gender effect | B6 effect | Interaction |
|-----------------------|--------|--------|---------------|-----------|-------------|
| Final body wt (g)     | 434±23*| 453±12a|               |           |             |
| Gains in body wt (g/6 wk) | 321±21b| 339±12a| <0.01         | <0.05     | 0.96        |
| Food intake           | 722±43a| 801±34a|               |           |             |
| Cecal contents wt (g) | 2.43±0.15b| 2.16±0.22a| 1.30±0.08b| 1.60±0.26ab| 1.47±0.11ab |
| Cecal contents wt/100 g body wt | 0.56±0.03| 0.48±0.05| 0.51±0.03| 0.58±0.09| 0.51±0.03 |
| Serum PLP (nmol/L)    | 0.17±0.07*| 0.55±0.02b| 1.14±0.03c| 0.05±0.00a| 0.45±0.02ab| 0.73±0.03b |

Mean±SE (n=8). Values with different superscripts are significantly different (p<0.05) by Tukey-Kramer HSD test.

**Table 3. Effects of gender difference and dietary B6 on fecal weight, IgA and mucins in rats.**

| Parameters            | Male   | Female | Gender effect | B6 effect | Interaction |
|-----------------------|--------|--------|---------------|-----------|-------------|
| Fecal dry wt (g/2 d/100 g body wt) | 4.01±0.20a| 4.24±0.31a| 2.68±0.14b| 3.02±0.11b| 3.23±0.12ab |
| Fecal IgA (mg/g dry feces) | 0.17±0.07| 0.22±0.04| 0.28±0.05| 0.26±0.04| 0.22±0.03| 0.20±0.02 |
| Fecal mucin (mg/g dry feces) | 0.11±0.03a| 0.19±0.03a| 0.20±0.02a| 0.41±0.06b| 0.78±0.07b| 1.01±0.13b |
| Fecal mucin (mg/2 d/100 g body wt) | 0.47±0.15*| 0.76±0.15*| 0.87±0.09a| 1.10±0.15a| 2.38±0.24a| 3.18±0.34b |

Mean±SE (n=8). Values with different superscripts are significantly different (p<0.05) by Tukey-Kramer HSD test.

Data were analyzed by the second derivative maximum method of the StepOne Real-Time PCR Software. The plasmid copy number per microliter was determined for standard plasmid solution [ng of cut standard plasmid mixture/μL×(molecules. bp/1.0×10^9 ng)×1/1660 DNA length bp per plasmid=plasmid copies/μL]. Real-time qPCR was run on serial dilutions of each standard mixture to relate threshold cycle number to copy numbers of the target sequence and to generate standard curves for quantification in unknown samples. Typically, standard curves were linear across 5 orders of magnitude (R^2>0.98).

**Fecal immunoglobulin A (IgA) analysis.** The analysis of fecal IgA was conducted by enzyme-linked immunosorbent assay (ELISA). For IgA extraction, 100 mg of freeze-dried fecal matter was homogenized on ice with 4 mL phosphate buffered saline (pH 7.2) containing 0.1 mg/mL soybean trypsin inhibitor (Wako), 50 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenized solution was incubated at 4°C overnight, and then centrifuged at 9,000 × g for 10 min. The supernatant was further used for assaying fecal IgA concentration, and stored at −70°C until analyzed. Quantification of fecal IgA was carried out using the Rat IgA ELISA Quantification Kit (Bethyl Laboratories, Montgomery, TX) according to the manufacturer’s protocol.

**Fecal mucin analysis.** The analysis of fecal mucins was conducted by fluorometric assay (45, 46). Briefly, each freeze-dried fecal sample of 100 mg was used for extraction and partial purification of mucin. O-Glycosid-
we used the fecal mucin assay kit (Cosmo Bio, Tokyo, Japan) according to the manufacturer’s protocol.

**Colonic gene expression analysis**. The analysis of colonic gene expression was done using real-time quantitative PCR (qPCR) using the StepOne™ Real-Time PCR System (Applied Biosystems). The Qiagen Midi kit (Qiagen AB, Hilden, Germany) was used to isolate total RNA (500 and 1,200 ng/μL) from rat colons according to the manufacturer’s protocol; silica-gel membrane and spin-column technology were used to efficiently remove the genomic DNA for the purification of total RNA. The reverse transcriptase reaction was carried out with 1 μg total RNA as a template to synthesize cDNA using

| Amino acids | Male | Female | Two-way ANOVA (p value) |
|-------------|------|--------|------------------------|
|             | B<sub>6</sub> (mg/kg diet) |         | Gender effect | B<sub>6</sub> effect | Interaction |
|             | 1 | 7 | 35 | 1 | 7 | 35 |
| Val (nmol/g tissue) | 43.5 ± 3.2 | 41.3 ± 3.9 | 45.7 ± 3.8 | 41.6 ± 3.4 | 40.6 ± 3.4 | 41.5 ± 5.5 | 0.53 | 0.23 | 0.10 |
| Leu (nmol/g tissue) | 47.3 ± 3.1 | 51.5 ± 3.0 | 51.4 ± 3.4 | 48.3 ± 3.5 | 43.2 ± 4.4 | 48.8 ± 4.7 | 0.36 | 0.72 | 0.44 |
| Ile (nmol/g tissue) | 24.0 ± 1.8 | 26.1 ± 1.3 | 26.7 ± 2.4 | 24.5 ± 1.5 | 20.4 ± 3.5 | 18.0 ± 4.4 | 0.11 | 0.74 | 0.20 |
| Met (nmol/g tissue) | 16.8 ± 3.6 | 20.2 ± 4.6 | 20.5 ± 3.5 | 16.9 ± 1.5 | 17.2 ± 3.1 | 19.3 ± 4.0 | 0.76 | 0.40 | 0.78 |
| Arg (nmol/g tissue) | 40.7 ± 4.0 | 44.3 ± 3.5 | 41.6 ± 3.0 | 41.2 ± 3.8 | 41.0 ± 3.1 | 38.1 ± 6.9 | 0.54 | 0.80 | 0.87 |
| Lys (nmol/g tissue) | 109 ± 6 | 115 ± 9 | 117 ± 10 | 129 ± 10 | 135 ± 10 | 129 ± 12 | 0.06 | 0.78 | 0.88 |
| Thr (nmol/g tissue) | 215 ± 9<sup>a</sup> | 273 ± 20<sup>a</sup> | 264 ± 18<sup>a</sup> | 295 ± 25<sup>a</sup> | 284 ± 23<sup>a</sup> | 336 ± 23<sup>b</sup> | <0.05 | 0.07 | 0.16 |
| Ala (nmol/g tissue) | 652 ± 33 | 721 ± 44 | 679 ± 16 | 663 ± 35 | 677 ± 35 | 704 ± 34 | 0.92 | 0.44 | 0.57 |
| Gly (nmol/g tissue) | 518 ± 44 | 564 ± 18 | 532 ± 84 | 466 ± 45 | 515 ± 42 | 527 ± 64 | 0.63 | 0.28 | 0.70 |
| Ser (nmol/g tissue) | 176 ± 7 | 213 ± 8 | 210 ± 14 | 194 ± 14 | 205 ± 14 | 226 ± 14 | 0.41 | 0.01 | 0.25 |
| Asn (nmol/g tissue) | 101 ± 20 | 122 ± 16 | 134 ± 14 | 83 ± 15 | 94 ± 15 | 100 ± 16 | 0.13 | 0.25 | 0.90 |
| Gln (nmol/g tissue) | 673 ± 27 | 745 ± 53 | 733 ± 54 | 632 ± 41 | 635 ± 33 | 642 ± 70 | 0.20 | 0.40 | 0.51 |
| Asp (nmol/g tissue) | 488 ± 21 | 572 ± 38 | 551 ± 18 | 630 ± 32 | 610 ± 43 | 612 ± 53 | 0.07 | 0.53 | 0.18 |
| Glu (nmol/g tissue) | 805 ± 29 | 907 ± 69 | 876 ± 30 | 902 ± 27 | 934 ± 42 | 919 ± 33 | 0.21 | 0.18 | 0.59 |
| Asn/Asp ratio | 0.21 ± 0.03 | 0.21 ± 0.02 | 0.24 ± 0.03 | 0.13 ± 0.02 | 0.15 ± 0.02 | 0.16 ± 0.02 | <0.05 | 0.28 | 0.81 |
| Gln/Glu ratio | 0.84 ± 0.05 | 0.83 ± 0.03 | 0.84 ± 0.05 | 0.70 ± 0.04 | 0.69 ± 0.05 | 0.69 ± 0.05 | <0.05 | 0.84 | 0.99 |
| Phe (nmol/g tissue) | 20.8 ± 2.2 | 24.2 ± 2.8 | 23.2 ± 2.5 | 19.6 ± 1.3 | 16.8 ± 1.7 | 20.8 ± 2.9 | 0.08 | 0.71 | 0.38 |
| Tyr (nmol/g tissue) | 28.4 ± 2.2<sup>a</sup> | 33.4 ± 2.9<sup>b</sup> | 31.6 ± 2.8<sup>b</sup> | 23.8 ± 2.3<sup>a</sup> | 21.7 ± 1.3<sup>a</sup> | 23.9 ± 1.9<sup>b</sup> | <0.01 | 0.31 | 0.11 |
| Phe/Tyr ratio | 0.73 ± 0.05 | 0.72 ± 0.05 | 0.69 ± 0.04 | 0.87 ± 0.10 | 0.77 ± 0.05 | 0.87 ± 0.09 | <0.05 | 0.72 | 0.63 |
| His (nmol/g tissue) | 15.6 ± 0.7 | 16.9 ± 1.1 | 14.9 ± 2.9 | 15.1 ± 1.8 | 14.3 ± 1.8 | 16.1 ± 1.2 | 0.67 | 0.98 | 0.38 |
| Tau (μmol/g tissue) | 2.44 ± 0.18 | 2.57 ± 0.11 | 2.62 ± 0.14 | 2.61 ± 0.12 | 2.59 ± 0.16 | 2.80 ± 0.12 | 0.47 | 0.14 | 0.61 |
| Urea (μmol/g tissue) | 6.21 ± 0.52 | 6.23 ± 0.63 | 5.95 ± 0.35 | 6.26 ± 0.60 | 6.81 ± 0.54 | 7.53 ± 0.72 | 0.28 | 0.49 | 0.20 |
| GABA (μmol/g tissue) | 5.39 ± 0.53 | 5.83 ± 0.61 | 5.38 ± 0.82 | 6.49 ± 0.93 | 6.27 ± 0.93 | 6.53 ± 1.01 | 0.33 | 0.98 | 0.84 |
| NH<sub>3</sub> (μmol/g tissue) | 0.95 ± 0.11 | 0.97 ± 0.12 | 1.02 ± 0.06 | 1.03 ± 0.05 | 0.92 ± 0.04 | 1.05 ± 0.07 | 0.83 | 0.42 | 0.61 |
| Orn (μmol/g tissue) | 4.8 ± 0.4 | 4.8 ± 0.4 | 4.6 ± 0.4 | 3.8 ± 0.3 | 3.6 ± 0.5 | 4.0 ± 0.2 | <0.05 | 0.87 | 0.62 |

Mean ± SE (n = 6). Values with different superscripts are significantly different (p < 0.05) by Tukey-Kramer HSD test.

GABA, γ-aminobutyric acid; Orn, ornithine; Tau, taurine.

**Fig. 1.** Correlation of fecal mucins with colon free threonine (A), serine (B) and gene expression of MUC16 (C).
ReverTra Ace (Toyobo) and random hexamers (TaKaRa Bio, Otsu, Japan) in a final reaction volume of 20 µL, according to the manufacturer’s protocol. Quantitative polymerase chain reaction (qPCR) was performed with a StepOneTM Real-Time PCR System (Applied Biosystems) using the Thunderbird SYBR qPCR Mix (Toyobo). Briefly, for qPCR analysis, SYBR-Green (containing DNA polymerase) of 10 µL forward and reverse primers (0.8 µM each), and 1,000 ng cDNA were contained in a final reaction volume of 20 µL. The cycling parameters were as follows: Initial step at 90°C for 10 s, followed by 40 cycles at 90°C for 5 s, 60°C for 10 s, and 72°C for 10 s. The mRNA level was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and all reactions were performed at least in duplicate. Analysis of relative gene expression levels was performed using the following formula: \( \Delta \Delta CT = CT_{target} - CT_{GAPDH} \) (target gene) – Cq (control) (47).

Statistical analysis. Data were expressed as the average ± standard error (SE). Two-way analysis of variance (ANOVA) and the Tukey-Kramer HSD test were used with \( p<0.05 \) being considered as significant. Some data underwent regression analysis and the correlation coefficient was calculated, and \( p<0.05 \) was considered statistically significant.

RESULTS

Body weights, food intake, and PLP

Final body weights, gains in body weight, and food intake were significantly higher in the male rats than in the female rats (Table 2, \( p<0.05 \), ANOVA). Additionally, serum PLP levels in the male rats were significantly higher than in the female rats (Table 2, \( p<0.05 \), ANOVA). Dietary supplemental B6 to the low B6 diet (1 mg PN HC1/kg diet) caused higher serum PLP levels in both male and female groups (\( p<0.05 \), ANOVA). There was no interaction of the effects of gender difference and dietary B6 on serum PLP, final body weight, gains in body weight, or food intake (\( p>0.05 \), ANOVA).
Table 6. Effects of gender difference and dietary B<sub>6</sub> on the abundance of microflora in feces of rats.

| Microflora                  | Male (B<sub>6</sub> mg/kg diet) | Female (B<sub>6</sub> mg/kg diet) | Two-way ANOVA (p value) |
|----------------------------|---------------------------------|-----------------------------------|------------------------|
|                            | 1  | 7  | 35 | 1  | 7  | 35 | Gender effect | B<sub>6</sub> effect | Interaction |
| Total bacteria (×10<sup>14</sup>) | 2.63±0.85  | 3.92±0.44  | 4.34±1.82  | 2.15±0.87  | 2.40±0.82  | 2.57±0.90  | 0.22  | 0.51  | 0.77  |
| Bifidobacterium spp. (×10<sup>10</sup>) | 0.36±0.16  | 0.49±0.25  | 1.31±0.97  | 0.85±0.62  | 0.01±0.00  | 0.02±0.02  | 0.28  | 0.67  | 0.22  |
| Lactobacillus spp. (×10<sup>13</sup>) | 4.56±1.85* | 3.82±0.62* | 6.06±2.79* | 2.18±1.61<sup>ab</sup> | 0.28±0.10<sup>b</sup> | 1.14±0.50<sup>b</sup> | <0.05 | 0.49  | 0.63  |
| Clostridium cocoides (×10<sup>13</sup>) | 4.86±2.11  | 5.56±1.81  | 7.28±1.7   | 5.96±2.80  | 1.04±0.38  | 2.65±0.93  | 0.12  | 0.43  | 0.17  |
| Clostridium leptum (×10<sup>13</sup>) | 0.81±0.38  | 1.64±0.32  | 1.54±0.92  | 0.29±0.11  | 2.54±1.00  | 1.07±0.59  | 0.95  | 0.06  | 0.44  |
| Akkermansia muciniphila (×10<sup>12</sup>) | 1.23±0.47  | 2.05±0.28  | 2.53±0.74  | 1.07±0.66  | 2.84±0.90  | 1.21±0.52  | 0.58  | 0.12  | 0.22  |
| Bacteroidetes (×10<sup>13</sup>) | 1.98±0.65  | 4.61±0.89  | 2.96±0.75  | 2.19±0.98  | 4.36±2.20  | 1.83±1.03  | 0.69  | 0.11  | 0.85  |
| Firmicutes (×10<sup>13</sup>) | 0.75±0.23  | 1.34±0.15  | 1.33±0.48  | 0.71±0.30  | 1.68±0.81  | 0.85±0.29  | 0.87  | 0.21  | 0.64  |

Mean±SE, n=7–8; *indicates studies using 7 animals and others are the data for 8 animals.
Values with different superscripts are significantly different (p<0.05) by Tukey-Kramer HSD test.

Fecal IgA and mucins

Fecal dry weights of the female rats were significantly lower than those of the male rats (Table 3, p<0.05, ANOVA). Fecal levels of IgA were unaffected. When expressed in both per g dry feces and per 2 d, fecal levels of mucins were significantly higher in the female rats than in the male rats (p<0.05, ANOVA). ANOVA showed dietary supplemental B<sub>6</sub> significantly increased fecal mucins (p<0.05), although the significant effect of B<sub>6</sub> was observed only in the female rats by the Tukey-Kramer HSD test (p<0.05). There was an interaction of the effects of gender difference and dietary B<sub>6</sub> on fecal mucins (p<0.05, ANOVA). The increase in fecal mucins by B<sub>6</sub> supplementation appeared to be prominent in the female groups. There was no correlation of serum PLP with fecal mucins per 2 d (r=0.26, p>0.05).

Colonie free amino acids

Colonial counts of free threonine were significantly higher in the female rats than in the male rats (Table 4, p<0.05, ANOVA), but were unaffected by dietary level of B<sub>6</sub>. Meanwhile, among the female groups, the threonine levels were significantly higher in the 35 mg PN HCl/kg diet than in the 1 and 7 mg PN HCl/kg diets (p<0.05, Tukey-Kramer HSD test). Colonial levels of free serine were significantly affected by dietary level of B<sub>6</sub> (p<0.05, ANOVA). Although the Tukey-Kramer HSD test indicated no significant effect of B<sub>6</sub> on the serine levels among the groups, the serine levels appeared to...
be higher in the 7 and 35 mg PN HCL/kg groups compared to the 1 mg PN HCL/kg group. Colon levels of free tyrosine were significantly higher in the male rats than in the female rats (p<0.05, ANOVA), but were unaffected by dietary B6 (p>0.05, ANOVA). Colon levels of free ornithine were slightly, but significantly, higher in the male rats than in the female rats (p<0.05, ANOVA), but were unaffected by dietary B6. The levels of other free amino acids were unaffected. There was a significant correlation of fecal mucins (per 2 d) with cecal *Bifidobacterium* spp. was markedly higher in the male rats than in the female rats (p<0.05, ANOVA), but was unaffected by dietary B6, whereas the numbers of cecal *Bifidobacterium* spp. were unaffected by both gender difference and dietary B6 (p>0.05, ANOVA). The numbers and the relative abundance (%) of cecal *Lactobacillus* spp. were both markedly higher in the male rats than in the female rats (Table 5, p<0.05, ANOVA), but were unaffected by dietary B6. The numbers of other microflora examined were unaffected. There was no interaction of the effects of gender difference and dietary B6 on the numbers of microflora examined (p>0.05, ANOVA). The relative abundance (%) of cecal *Bacteroides* was markedly higher in the female rats than in the male rats (p<0.05, ANOVA). The numbers and relative abundance of cecal *Akkermansia muciniphila* were unaffected. **Fecal microflora**

As shown in Table 6, the relative abundance (%) of cecal *Bifidobacterium* spp. was markedly higher in the male rats than in the female rats (p<0.05, ANOVA), but was unaffected by dietary B6, whereas the numbers of

### Table 7. Effects of gender difference and dietary B6 on relative expression of colonic genes of mucins in rats.

| Gene   | Male | B6 (mg/kg) diet | Female | Two-way ANOVA (p value) |
|--------|------|-----------------|--------|-------------------------|
|        | 1    | 7   | 35 | 1 | 7 | 35 | Gender effect | B6 effect | Interaction |
| MUC1   | 0.89±0.09* | 0.88±0.14* | 0.76±0.14* | 0.54±0.05 | 0.51±0.06 | 0.58±0.09 | 0.09 | 0.95 | 0.70 |
| MUC2   | 0.46±0.15* | 0.52±0.19* | 0.50±0.19* | 0.14±0.05 | 0.18±0.06 | 0.14±0.05 | 0.06 | 0.92 | 0.98 |
| MUC3   | 0.79±0.26* | 0.60±0.22* | 0.74±0.22* | 0.22±0.08 | 0.20±0.07 | 0.17±0.08 | <0.05 | 0.84 | 0.84 |
| MUC4   | 1.34±0.47* | 0.93±0.18* | 0.95±0.24* | 1.42±0.08 | 0.94±0.07 | 0.67±0.07 | 0.82 | 0.87 | 0.83 |
| MUC5AC | 1.49±0.19* | 1.80±0.36* | 1.30±0.24* | 1.38±0.14 | 1.27±0.19 | 1.25±0.19 | 0.85 | 0.71 | 0.71 |
| MUC6   | 0.89±0.04**ab | 0.91±0.20**ab | 1.08±0.15**ab | 1.27±0.20* | 0.74±0.05b | 0.67±0.07b | 0.37 | 0.29 | <0.03 |
| MUC7   | 0.70±0.10* | 1.15±0.25* | 0.57±0.12* | 0.48±0.07 | 0.63±0.19 | 0.85±0.27 | 0.79 | 0.26 | 0.09 |
| MUC8   | 1.04±0.13**ab | 1.67±0.23**ab | 0.96±0.15**ab | 1.26±0.08ab | 1.61±0.15ab | 1.27±0.17ab | <0.05 | <0.05 | 0.74 |
| MUC9   | 0.84±0.05* | 0.93±0.08* | 0.74±0.14* | 0.96±0.06 | 1.09±0.09 | 1.09±0.11 | <0.01 | 0.71 | 0.68 |

Mean±SE. n=6–8; * and ** indicate studies using 7 and 6 animals, respectively, and others are the data for 8 animals. Values with different superscripts are significantly different (p<0.05) by Tukey-Kramer HSD test.

### Table 8. Effects of gender difference and dietary B6 on relative expression of colonic genes of TLRs in rats.

| Genes | Male | B6 (mg/kg) diet | Female | Two-way ANOVA (p value) |
|-------|------|-----------------|--------|-------------------------|
|       | 1    | 7   | 35 | 1 | 7 | 35 | Gender effect | B6 effect | Interaction |
| TLR1  | 1.28±0.30* | 1.55±0.35* | 1.07±0.36* | 1.22±0.22 | 0.75±0.11 | 0.68±0.16 | 0.30 | 0.36 | 0.39 |
| TLR2  | 1.00±0.30* | 1.32±0.30* | 0.27±0.47* | 1.08±0.17 | 0.69±0.15 | 0.82±0.16 | 0.48 | 0.98 | 0.39 |
| TLR3  | 0.60±0.19* | 0.64±0.22* | 0.54±0.16* | 0.39±0.08 | 0.41±0.11 | 0.06±0.36 | 0.83 | 0.87 | 0.64 |
| TLR4  | 1.03±0.27* | 1.32±0.44* | 1.07±0.19* | 0.67±0.08 | 0.75±0.30 | 0.86±0.34 | 0.35 | 0.79 | 0.79 |
| TLR5  | 0.78±0.23* | 1.03±0.43* | 0.95±0.31* | 0.64±0.17 | 0.64±0.19 | 0.88±0.54 | 0.78 | 0.80 | 0.86 |
| TLR6  | 1.13±0.07* | 2.24±0.62* | 1.35±0.27* | 1.44±0.19 | 1.17±0.16 | 1.26±0.10 | 0.78 | 0.39 | 0.10 |
| TLR7  | 2.04±1.31** | 2.78±0.93* | 2.10±1.29* | 2.26±1.05 | 1.19±0.52 | 1.02±0.38 | 0.60 | 0.81 | 0.42 |
| TLR8  | 1.49±0.30** | 2.70±0.98**ab | 2.07±0.50**ab | 4.03±0.44**b | 3.46±0.48**ab | 2.75±2.01**b | <0.01 | 0.46 | 0.16 |
| TLR9  | 1.56±0.72* | 2.67±0.99* | 1.91±0.90* | 1.94±0.60 | 0.93±0.35 | 0.96±0.19 | 0.44 | 0.78 | 0.20 |

Mean±SE. n=6–8; * and ** indicate studies using 7 and 6 animals, respectively, and others are the data for 8 animals. Values with different superscripts are significantly different (p<0.05) by Tukey-Kramer HSD test.
fecal *Bifidobacterium* spp. were unaffected by both gender difference and dietary B6 (p > 0.05, ANOVA). The numbers and the relative abundance (%) of fecal *Lactobacillus* spp. were both markedly higher in the male rats than in the female rats (Table 6, p < 0.05, ANOVA), but were unaffected by dietary B6. The numbers of other microflora examined were unaffected. There was no interaction of the effects of gender difference and dietary B6 on the numbers of microflora examined (p > 0.05, ANOVA). The relative abundance (%) of fecal *C. cocolides* and *Firmicutes* was significantly affected by dietary B6, but was unaffected by gender difference. The relative abundance of fecal *C. cocolides* in the 7 mg PN HCl/kg male and female rats appeared to be higher than that in the 1 and 35 mg PN HCl/kg male and female rats (p < 0.05, Tukey-Kramer HSD test). Meanwhile, the relative abundance of fecal *Firmicutes* in the 1 mg PN HCl/kg female rats was significantly lower than in the 35 mg PN HCl/kg female rats (p < 0.05, Tukey-Kramer HSD test). The numbers and relative abundance of fecal *Akkermansia muciniphila* were unaffected.

**Colonie gene expression of mucins (MUCs)**

As shown in Table 7, the gene expression of MUC16 was significantly higher in the female rats than in the male rats (p < 0.01, ANOVA), but was unaffected by dietary B6. Fecal levels of mucins (per 2 d) were significantly associated with the gene expression of MUC16 (r = 0.47, p < 0.01, Fig. 1C), but not with those of other MUCs (p > 0.05).

**Colonie gene expression of toll-like receptors (TLRs)**

The gene expression of TLR8 was significantly higher in the female rats than in the male rats (Table 8, p < 0.05, ANOVA). In particular, the effect of gender difference on the expression of TLR8 was prominent in the 1 mg PN HCl/kg female rats (p < 0.05, Tukey-Kramer HSD test). The gene expressions of the other TLRs were unaffected.

**DISCUSSION**

Our previous study indicated that supplementation of B6 to a 30% beef tallow diet resulted in higher fecal mucins in the male rats (48), whereas the present study indicated no significant increase in the fecal mucins in the male rats that were given a 20% corn oil diet. Nevertheless, ANOVA showed the overall increase in the fecal mucins by supplemental B6 in both the male and the female rats. This study further indicated a significant interaction of the effects of gender difference and dietary B6 on the fecal mucin. In other words, the increase of the fecal mucin levels in the female rats by dietary B6 was more profound than in the male rats. At the same time, this study found no effect of gender and B6 on fecal IgA. Mucins have the unique function of protecting and lubricating epithelial surfaces, but they are implicated in additional diverse roles such as growth, fetal development, epithelial renewal and differentiation, epithelial integrity, carcinogenesis, and metastases (49). Thus, dietary B6 and gender may modulate the colon luminal environment through mucin production, but not IgA production. However, the physiological implication of the effect of B6 and gender difference on fecal mucins remains to be investigated.

Supplemental threonine has been suggested to enhance the intestinal mucin production in rats (50, 51). Consumption of an amino acid mixture containing L-threonine, L-serine, L-proline, and L-cysteine has been also suggested to promote mucin synthesis (52). Therefore, in this study, colonic free amino acids were determined in order to learn the effect of threonine on mucin production. As a result, this study indicated that the modulation of colonic free threonine and serine by gender and B6 was associated with the alteration in the fecal mucin levels. Thus, the effect of gender and dietary B6 on fecal mucin levels may be related to the alterations in colonic free threonine and serine. Intriguingly, the female rats had higher levels of free threonine in the colon compared with the male rats. To our knowledge, this is the first evidence of the effect of gender difference on colonic threonine in an animal study. Rémond et al. (53) reported that inflammation stimulated intestinal mucin production and threonine uptake in minipigs. Thus, it is of interest to test whether higher inflammation in female animals is related to higher fecal mucins and colonic threonine. Liu et al. (54) reported that threonine has an important role in intestinal mucosal protein synthesis, particularly mucin, and in intestinal integrity, immune barrier function, and oxidative stress. The present study further revealed the increase of free serine in the colon by dietary supplemental B6. A recent meta-omics study by Ramos et al. (55) showed lower serine concentrations in B6-deprived Neuro-2a cells, implying that B6 was essential for serine de novo biosynthesis in the brain. Thus, further study is necessary to examine whether higher free serine in the colon from dietary B6 supplementation can be ascribed to higher biosynthesis.

This study indicated that colonic free ornithine was significantly lower in the female rats than in the male rats, although it was unaffected by dietary B6. Previous studies have indicated that high ornithine decarboxylase (ODC) activity is associated with colon diseases such as UC and CD (56) and with the malignant state of rat and human colons (57). Recently, Hardbower et al. (58) found that ODC activates M1 macrophage and causes inflammation. Thus, it is of interest to test whether the lower ornithine in the female colon is related to higher ODC activity and higher inflammation.

This study further indicated that Asn/Asp ratio and Gln/Glu ratio were both significantly lower in the female rats than in the male rats. Asparagine and glutamine have been reported to stimulate ODC activity and cell proliferation (59, 60). Asparagine and glutamine serve as primary nutrients for the intestine by providing nitrogen, which is necessary for DNA synthesis or cell proliferation. Thus, it is necessary to investigate whether a lower Asn/Asp ratio and lower Gln/Glu ratio in female rats is associated with higher utilization or catabolism of asparagine and glutamine, which in turn leads to higher ODC activity and lower ornithine levels. On the other hand, asparagine has been reported to preserve intestinal barrier function and improve intestinal integrity in inflammatory in weanling piglets (59, 61).
whereas glutamine decreased the LPS-induced inflammatory response in infant rat intestines (62). Thus, further study is necessary to test whether a lower Asn/Asp ratio and Gln/Glu ratio in the female rats causes lower intestinal integrity and higher inflammation. In addition, our study indicated a higher Phe/Tyr ratio in the male rats than in the male rats. It has been reported that the inflammatory state during infection is associated with the serum Phe/Tyr ratio (63). Thus, this result raises the question whether the implication of a higher Phe/Tyr ratio in the colon of female rats is related to inflammation.

Yurkovetsyskiy et al. (64) reported that male mice have a higher relative abundance of cecal Lactobacillus compared with female mice or castrated male mice. However, Fransen et al. (65) indicated that conventional female mice had a higher relative abundance of fecal Lactobacillus spp. than conventional male mice. Our recent study indicated that the alteration in fecal Lactobacillus spp. by some dietary manipulation was not necessarily consistent with that in cecal Lactobacillus spp. (48). Therefore, we examined both cecal and fecal microflora in rats. The results showed a higher relative abundance and numbers of cecal and fecal Lactobacillus spp. in the male rats than in the female rats, which is in agreement with those by Yurkovetsyskiy et al. (64). It is unknown why our results were not consistent with the findings of Fransen et al. (65). Zhang et al. (66) have reported that the relative abundance of fecal Bifidobacterium spp. is higher in male rats than in female rats; consistent with Zhang et al.’s study (66), our work also found that the relative abundance of cecal and fecal Bifidobacterium spp. was significantly higher in the male rats than in the female rats. Our results implied that the levels of beneficial bacteria such as Lactobacillus spp. and Bifidobacterium spp. were both affected by gender difference. Such beneficial bacteria have been reported to have an important role in gastrointestinal inflammation. Liu et al. (67) has reported that human-derived probiotic Lactobacillus reuteri strains reduce intestinal inflammation. Oral treatment with Bifidobacterium longum 51A reduces inflammation in a murine experimental model of gout (68). Therefore, it is worthwhile to test the possibility that the lower abundance of these beneficial bacteria in female rats may be associated with higher inflammation.

In relation to the degradation of mucins, intestinal Akkermansia muciniphila (mucin degrader) has an important role. The present study indicated no influence of gender and dietary B6 on the abundance of cecal or fecal Akkermansia muciniphila. Thus, the results did not support the possibility of the involvement of Akkermansia muciniphila in the mechanism of the combined effect of gender and B6 on fecal mucins. However, it remains to determine the activity of mucin-degrading enzyme (mucinase) to test the possibility of the involvement of mucin degradation. Recent studies have suggested that other intestinal bacteria such as some strains of Bifidobacterium, Bacteroides, and Ruminococcus other than Akkermansia muciniphila are also able to degrade mucins (69, 70). Therefore, the possibility of the involvement of such bacteria in the alteration of mucins cannot be ruled out.

This study indicated that the gene expression of MUC16 was significantly higher in the female rats than in the male rats, although this expression was unaffected by dietary B6. Importantly, we found that the gene expression of MUC16 had a significant positive correlation with the fecal mucins. Thus, the effect of gender difference on the fecal mucins might be mediated by the change in the gene expression of MUC16. The expressions of MUC3 were also affected by gender difference. However, the implication of such alterations is unknown.

Since MUC expression is closely associated with TLR expression (71), this study further examined the gene expression of TLRs. As a result, this study indicated that TLR8 expression was significantly higher in the female rats than in the male rats, regardless of dietary B6. The function of TLR8 in epithelial cells is to induce pro-inflammatory signals (72). TLR8 expression has been associated with colon diseases such as CD (73, 74), UC (74, 75), CRC (76), IBD (77). Therefore, it might be possible that TLR8 expression is related to the incidence of gender-dependent colon diseases. However, further study is necessary to test this possibility.

In conclusion, we found a combined effect of gender and dietary B6 on fecal mucin levels. Such an effect was suggested to be mediated by the alterations in colonic free threonine and serine and in MUC16 expression. Furthermore, gender difference significantly affected several colonic parameters such as free amino acids, including threonine, ornithine. Asn/Asp ratio, and Gln/Glu ratio, abundance of Lactobacillus spp., and expressions of MUC16 and TLR8, as being important to colon health and diseases. This study suggests that gender difference and dietary B6 have a significant impact on the colon luminal environment by modulating these parameters. Further study is necessary to investigate if such alterations in the parameters are related to sex hormones.

Author contribution
NK designed the research; DN, YY, TK, and NY conducted the research; DN analyzed the data; and DN, TK, NY and NK wrote the paper and had primary responsibility for the final content. All authors read and approved the final manuscript.

Acknowledgments
The authors would like to thank Enago (www.enago.jp) for the English language review.

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