

# Intensive, prolonged exercise seemingly causes gut dysbiosis in female endurance runners

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Intensive, prolonged exercise is known to induce gastrointestinal disorders such as diarrhea, with gut dysbiosis suggested as being one of the causatives. In the present study, we wanted to investigate the relationship between intensive exercise and the gut microbiota status. To that end, the microbiota, the moisture content and the bacterial metabolites (e.g., organic acids) of female endurance runners ( $n = 15$ ) and those of non-athletic but healthy, age-matching female controls ( $n = 14$ ) were compared. The analysis of the gut microbiota analysis showed that, unlike control subjects, female endurance runners had distinct microbiotas, with some bacteria found in higher abundances likely being involved in gut inflammation. The concentration of succinate, a gut bacterial metabolite regarded as undesirable when accumulated in the lumen, was significantly ( $p < 0.05$ ) higher in the female endurance runners. *Faecalibacterium*, that was significantly ( $p < 0.05$ ) abundant in female endurance runners, can produce succinate in certain environments and hence may contribute to succinate accumulation, at least partly. The present work suggested that the gut microbiotas of female endurance runners are seemingly dysbiotic when compared with those of control subjects. Further investigation of the mechanism by which intensive, prolonged exercise affects the gut microbiota is recommended.

**Key Words:** exercise-induced gastrointestinal disorders, gut microbiota, organic acids, putrefactive metabolites, female endurance runner

Athletes are more predisposed to experience of gastrointestinal disorders after practices and competition, which is known as exercise-induced gastrointestinal syndrome.<sup>(1,2)</sup> Although frequency of occurrence of the gastrointestinal syndrome differs depending on various factors such as the type of sport, intensity/duration of the exercise and gender, typical symptoms often observed are diarrhea, loose stool, abdominal pain, and/or intestinal bleeding in the lower digestive tract.<sup>(2-4)</sup> However, the mechanism of this disorder are yet to be fully elucidated. According to Costa *et al.*,<sup>(2)</sup> two primary pathways are suggested as causatives of this disorder. One of such pathways is the circulatory-gastrointestinal pathway, in which prolonged high-intensity exercise causes gut ischemia-reperfusion that is the factor of site-specific oxidative stress and intestinal injury.<sup>(3,5)</sup> The other is the neuroendocrine-gastrointestinal pathway, in which both physical and psychological stresses alter gut motility and transit through enteric nervous activity, which consequently causes gut malabsorption of nutrients.<sup>(6,7)</sup> Indeed, previous work has showed that intense endurance running causes

malabsorption of carbohydrates in triathletes.<sup>(8)</sup>

Changes in the gut microbiota caused by intensive exercise may also contribute to exercise-induced gastrointestinal disorders. For example, it has been reviewed elsewhere that gut dysbiosis, in other words, aberrant microbiota composition showing abnormal profiles of microbiota metabolites, can cause various gut disorders such as constipation, diarrhea and gut inflammation.<sup>(9,10)</sup>

In general, adequate exercise is regarded as beneficial for the gut.<sup>(11,12)</sup> For example, the abundances of butyrate-producing bacteria and concentrations of butyrate were observed to increase in humans after physical exercise.<sup>(13,14)</sup> However, this does not seem to be the case when the exercise is highly intensive and/or is physiologically stressful. Using a murine model, Allen *et al.*<sup>(15)</sup> reported that the effect on the gut microbiota is different when the exercise is voluntary or forced. Allen *et al.* found that voluntary exercise was beneficial for the gut health but forced exercise was not, because while the former attenuated gut inflammation, the latter further exacerbated it. Nonetheless, when compared with those of people living a regular lifestyle, the effect of highly-intensive exercise on the gut microbiotas of athletes remains unclear.<sup>(16,17)</sup>

In the present study, we aimed to obtain a basic understanding of the effect of highly-intensive endurance exercise on the gut microbiota for the possible development of future treatments of exercise-induced gastrointestinal disorders. We compared the gut environment, that is, the gut microbiota and its metabolites, between female elite endurance runners and non-athletic, but healthy women of similar age living within close geographic locations. In addition, fecal microbiota composition and three gut environmental parameters, namely the moisture content, the concentrations of organic acids and the concentrations of putrefactive metabolites were analyzed.

## Materials and Methods

**Ethics statements.** The experiment was approved by the Ethical Committee of Kyoto Prefectural University and conducted as per their guidelines (approval number: 148). A written informed consent was obtained from all participants.

**Research subjects.** Freshly evacuated feces were collected from female Japanese elite endurance runners belonging to a company team [endurance runners (ER) group:  $n = 15$ , monthly

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**Table 1.** Profiles of enrolled subjects

Variable	Control	ER	<i>p</i> value
Total <i>n</i> (male/female)	14 (0/14)	15 (0/15)	
Age (years)	20.9 ± 0.3	20.5 ± 1.2	0.80
Self reported BMI	21.3 ± 0.6	17.8 ± 0.2	<0.01**

\*\**p*<0.01.

running distance; 547 ± 111 km] and healthy female volunteers living in similar geographic location (control group: *n* = 14) (Table 1). All fecal specimens were collected from April 2018 to April 2019. There was no restriction on the timing and situation of fecal collection. The feces were collected using a fecal collection tube with a scoop (Sarstedt K.K., Tokyo, Japan) and immediately stored at -20°C or lower until further processing. It was not possible to analyze the fecal moisture content and the concentrations of putrefactive metabolites in one sample from the ER group because the fecal amount provided by the subject was too small. Therefore, for the analyses of fecal moisture content and putrefactive metabolites, the number of samples available from the ER group was 14.

#### Analysis of fecal microbiota by 16S rRNA metagenomics.

Extraction of bacterial DNA from feces, library preparation and deep sequencing (MiSeq, Illumina K.K., Tokyo, Japan) were carried out exactly as described by Inoue *et al.*<sup>(18)</sup> Sequence data analysis was carried out as described by Hashimoto *et al.*<sup>(19)</sup> using QIIME 2.<sup>(20)</sup>

**Measurement of the fecal moisture content.** Fecal moisture contents were determined by lyophilization as described elsewhere.<sup>(21)</sup>

#### Analysis of the concentrations of fecal organic acids.

The concentrations of fecal organic acids such as succinate, lactate, formate, acetate, propionate, iso-butyrate, *n*-butyrate, iso-valerate and *n*-valerate were measured by an ion-exclusion high-performance liquid chromatography as described elsewhere.<sup>(22)</sup>

**Analysis of the concentrations of fecal putrefactive metabolites.** Fecal putrefactive metabolites such as phenol, indole, skatole and para-cresol were measured by gas chromatography-mass spectrometry. Briefly, feces (100 mg) were thawed and transferred to 2 ml screw-cap tubes (Watson Co. Ltd., Tokyo, Japan) with a φ5.5 stainless steel ball (TOMY SEIKO Co. Ltd., Tokyo, Japan). Methanol (0.9 ml) and 10 μl of an internal standard methanol solution [1 mmol/L phenol-d5 (Sigma-Aldrich Japan, Tokyo, Japan)] were added and the feces were homogenized using Micro Smash MS-100 (3,000 r.p.m., 30 s). After centrifugation (15,000 × *g*; 5 min; 4°C), the supernatant was collected into a new 3.5 ml screw-capped cryotube (TPP Techno Plastic Products, Trasadingen, Switzerland). The residues were re-suspended in 1 ml of methanol, homogenized, centrifuged, and the supernatant was collected again as described above. This procedure was conducted two times. Total collected supernatants (approximately 2.9 ml) were mixed and used as samples for further analysis.

The method for the analysis of putrefactive metabolites was that described by GL Sciences Inc. (Tokyo, Japan; <https://www.gls.co.jp/viewfile/?p=GA119>). Briefly, the analysis was conducted using a gas chromatography mass spectrometry (GCMS-QP2010 Ultra; Shimadzu, Kyoto, Japan) equipped with an autosampler (AOC-5000; Shimadzu, Kyoto, Japan). Chromatographic separation was carried out with an InertCap5 column (30 m × 0.25 mm I.D. × 0.25 μm; GL Sciences Inc.). Helium was used as the carrier gas, and the linear velocity was set at 48.1 ml s. The stepwise thermal conditions of the column oven were as follows: the temperature was initially raised to and maintained at 40°C for 2 min; then it was raised to 320°C at a rate of 10°C/min and maintained for 10 min. The total run time of the analysis was 40 min. The mass spectrometer was set in selected ion monitoring

mode at *m/z* 117.0 of indole, *m/z* 108.0 of para-cresol, *m/z* 94.0 of phenol, *m/z* 99.0 of phenol-d5, and *m/z* 131.0 of skatole. The injection was set in split mode (10.0) with an injection volume of 1 μl and an injector temperature of 250°C. The chemical ionization mode was set as follows: an ion source temperature of 200°C and an interface temperature of 250°C. The concentration of each parameter was calculated by the peak area of the parameter and the peak area of the internal standard (phenol-d5).

**Statistical analysis.** α-Diversity indices Chao1 (richness) and Shannon (evenness) were calculated with the R phyloseq package.<sup>(23)</sup> β-Diversity was estimated based on UniFrac distances by QIIME 1.9.1 and tested by a principal coordinate analysis (PCoA). The UniFrac distance between samples was statistically analyzed by a permutational multivariate analysis of variance (PERMANOVA) by QIIME 1.9.1. Age and BMI were statistically compared by the Welch's *t* test. α-Diversity indices, the relative abundance (%) of bacterial phyla and genera, the fecal moisture content, and the concentrations of organic acids and putrefactive metabolites were statistically compared by the Wilcoxon's rank sum test. Differences between the means were considered to be significant if *p*<0.05 and with a tendency to be significant if 0.05<*p*<0.1. Values are expressed as the means ± SE. All statistical analyses were calculated by R (ver. 3.6.0), unless otherwise specified.

## Results

**Diversity analysis of the gut microbiota.** In the alpha-diversity analysis, the Chao1 index of the ER group showed a higher value (178 ± 13) than the control group (131 ± 35), although the difference was not significant (*p* = 0.09, Fig. 1). Similarly, no significant differences in the Shannon index were found between the two groups (*p* = 0.48). In contrast, the β-diversity between two groups was found to be significantly (*p*<0.05) different (Fig. 2). In the taxonomy analysis at the phylum level, the abundance of Deferribacteres was significantly higher in the ER group (0.006 ± 0.002%) than in the control group (0%). In addition, the abundance of OD-1 was also significantly higher in ER group (0.904 ± 0.167%) than in the control group (<0.001%) (Fig. 3). At the genus level, the relative abundances of 16 bacterial genera were significantly different between the two experimental groups (Table 2). For example, the abundance of 11 bacterial genera were significantly higher in the ER group than in the control group. In particular, the relative abundance of genera *Faecalibacterium* was more than 2-fold higher in the ER group (13.83 ± 1.55%) than in the control group (6.20 ± 1.14%).

#### The moisture content, and the concentrations of organic acids and putrefactive metabolites in feces (Table 3).

There was no significant difference in the fecal moisture content between the two experimental groups. The concentration of fecal succinate was significantly higher in the ER group (3.56 ± 1.41 mmol/kg wet feces) than in the control group (1.75 ± 1.08 mmol/kg wet feces). The concentration of fecal indole tended to be lower (*p* = 0.09), in the ER group (38.95 ± 11.68 μg/g wet feces) than in the control group (63.23 ± 13.58 μg/g wet feces). As for the other organic acids and putrefactive metabolites, no significant differences in the concentrations were found between the two experimental groups.

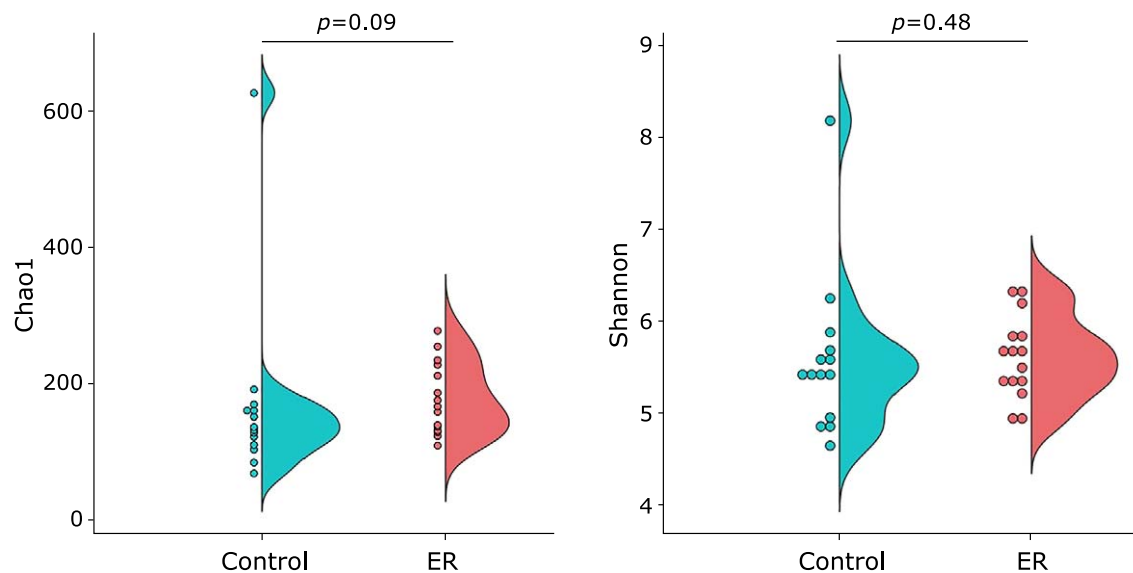


Fig. 1. Chao1 and Shannon indices of the gut microbiota.

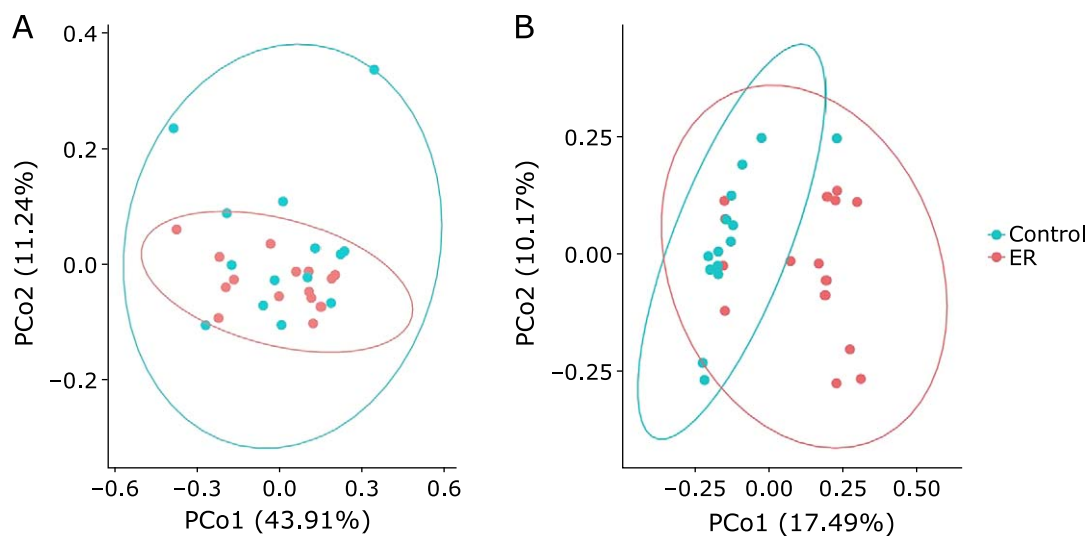


Fig. 2. PCoA (principal coordinate analysis) based on weighted UniFrac (A) and unweighted UniFrac distances (B) generated from the data of the fecal microbiota. The ellipse enclosing each cluster indicate a 95% confidence interval. The PERMANOVA analysis indicated that the gut microbiota compositions of the ER and control groups were different, based on the unweighted UniFrac ( $p < 0.05$ ), but not on the weighted UniFrac distance.

## Discussion

In the present work, we showed that gut microbiotas in the ER group, that is, the female elite endurance runners, were different from those in the control group. No significant differences were found in the Chao1 and Shannon indices between the two experimental groups (Fig. 1). However, one subject in the control group showed abnormally high values in both indices (Chao1: 627; Shannon: 8.18), which were regarded as outliers by the Smirnov-Grubbs test. When the values of this subject were excluded from the statistical comparison, the Chao1 index was significantly ( $p = 0.03$ ) higher in the ER group ( $178 \pm 13$ ) than in the control group ( $133 \pm 4$ ). Therefore, we believed that the ER group may have had a higher richness in their gut microbiotas than did the

control group. Nonetheless, this significance was not observed in the Shannon index even after excluding the outlying value ( $p = 0.27$ ). Separately, the PCoA based on the unweighted UniFrac distance, also showed that the composition of the gut microbiotas in the ER group were different than those in control group (Fig. 2; PERMANOVA;  $p < 0.05$ ).

The taxonomy analysis detected that some bacteria, which have been suggested as being associated with gut inflammation, were more abundant in the ER group than in the control group. For instance, genus *Haemophilus* and *Rothia* were reported to be abundant in Crohn's disease patients.<sup>(24)</sup> In addition, *Mucispirillum*, a mucus degradation bacterium, was found to be abundant in a murine inflammation model.<sup>(25,26)</sup> Similarly, *Ruminococcus gnavus*,<sup>(27)</sup> another mucus degradation bacterium, is reported to

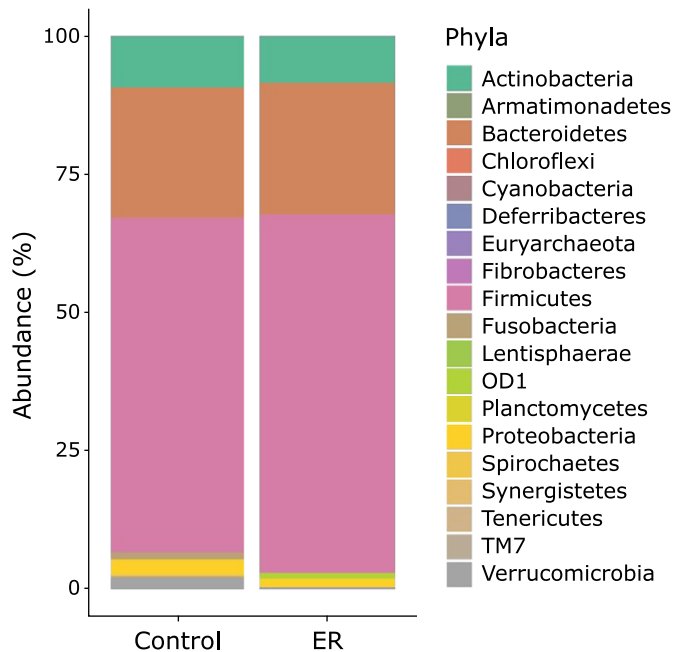


Fig. 3. The relative abundance of gut bacteria at the phylum level.

increase in the gut of mice when exercise was forcibly imposed, but not when it was voluntarily done by the animals.<sup>(15)</sup> Therefore, intensive exercise likely affects the production and/or sugar-chain composition of mucus in the gut.

By contrast, *Faecalibacterium*, which was also significantly ( $p < 0.05$ ) abundant in the ER group, is a butyrate-producing bacterium that is generally known to be beneficial for the host's health,<sup>(28,29)</sup> especially for its anti-inflammatory effect on the gut.<sup>(30)</sup> Therefore, in the present work, a higher abundance of *Faecalibacterium* was seemingly unlikely when possible proinflammatory bacteria such as *Mucispirillum* and family *Pasteurellaceae* were also present in high abundance, if a gut inflammation status is considered. However, Hansen *et al.*<sup>(31)</sup> pointed out that *Faecalibacterium* may have a more complex

role in IBD (inflammatory bowel disease) that goes beyond our current understanding, because they found a high abundance of *Faecalibacterium* in biopsy samples of inflamed tissues from IBD children. Moreover, in the present study, a high abundance of *Faecalibacterium* may have not necessarily been acting as a health-promoting bacterium, but instead, as an opportunistic microorganism, due to the altered gut environment of the ER group.

It is worth noting that *Faecalibacterium* is known for its succinate production. Indeed, *Faecalibacterium* produces succinate in gut environments rich in fumarate, for instance.<sup>(32)</sup> Under normal conditions, the succinate produced by gut bacteria is quickly metabolized to propionate, but it can accumulate under abnormal conditions.<sup>(33)</sup> For example, some *Bacteroides* species such as *B. fragilis* produce propionate via succinate, but this pathway does not work well in an environment with excessive carbohydrates or lack of vitamin B12. As a result, succinate is not metabolized and accumulated.<sup>(34,35)</sup> In the present study, a plausible explanation for the significantly higher concentration of succinate may be due to the abnormal metabolic pathway of *Faecalibacterium* in the microbiota of the ER group, probably affected by the intensive, prolonged exercise. It has been suggested that exercise increases the abundances of butyrate-producing bacteria such as *Faecalibacterium* and *Roseburia* in human subjects.<sup>(13,14)</sup> Although, our results showed a high abundance of *Faecalibacterium*, which is in agreement with previous work, no increase in butyrate was observed. This apparent discrepancy may be explained by the fact that the abnormal gut environment of the ER group led *Faecalibacterium* to produce succinate, but not butyrate.

The accumulation of succinate seems to increase the osmotic pressure in the lumen and to reduce the water absorption rate of the intestine.<sup>(36)</sup> Therefore, succinate accumulation may be a risk factor for diarrhea or loose stool. In addition, the association of succinate accumulation with gut inflammation have been previously reported.<sup>(37)</sup> Therefore, at least partly, succinate accumulation is likely a causative factor of exercise-induced gastrointestinal disorders.

The analysis of fecal putrefactive metabolites showed that the concentration of indole tended to be lower in the ER group (Table 3). The concentration of the putrefactive metabolites in the feces generally counteract the moisture content and the concentrations of organic acids.<sup>(38)</sup> Thus, we theorize that, unlike that of the control group, the gut environment of the ER group was prone to

Table 2. List of bacterial genus that significantly different abundance between control and ER group

Nearest known bacterial genus	Abundance (%)		
	Control	ER	p value
g_ <i>Acinetobacter</i>	<0.01	0.01 ± 0.00	0.001**
g_ <i>Actinomyces</i>	0.16 ± 0.09	0.02 ± 0.01	0.004**
g_ <i>Bacillus</i>	0.01 ± 0.01	0.06 ± 0.02	0.037*
g_ <i>Bilophila</i>	0.18 ± 0.05	0.04 ± 0.03	0.033*
g_ <i>Citrobacter</i>	0.16 ± 0.14	<0.01	0.032*
g_ <i>Clostridium</i>	0.32 ± 0.10	0.87 ± 0.17	0.017*
g_ <i>Faecalibacterium</i>	6.20 ± 1.14	13.83 ± 1.55	0.001**
g_ <i>Haemophilus</i>	0.02 ± 0.02	1.01 ± 0.40	0.008**
g_ <i>Lachnospira</i>	0.69 ± 0.36	1.94 ± 0.36	0.001**
g_ <i>Mucispirillum</i>	<0.01	0.01 ± 0.00	0.005**
f_ <i>Paraprevotellaceae</i> g_ <i>Prevotella</i>	0.13 ± 0.13	0.01 ± 0.00	0.004**
g_ <i>Rothia</i>	<0.01	0.03 ± 0.01	0.001**
Unclassified p_OD1	<0.01	0.90 ± 0.17	<0.001**
Unclassified f_ <i>Clostridiaceae</i>	<0.01	0.05 ± 0.04	0.022*
Unclassified f_ <i>Pasteurellaceae</i>	<0.01	0.01 ± 0.01	0.045*
Unclassified f_S24-7	0.06 ± 0.06	0.02 ± 0.01	0.019*

\* $p < 0.05$ , \*\* $p < 0.01$ .

**Table 3.** Moisture content, organic acid concentrations and putrefactive metabolites concentrations in the feces

Measurements	Control	ER	p value
Moisture content (%)	75.97 ± 2.32	76.62 ± 2.54	0.77
Organic acids (mmol/kg of wet feces)			
Succinate	1.75 ± 1.08	3.56 ± 1.41	0.03*
Lactate	0.06 ± 0.06	0.50 ± 0.32	0.29
Formate	0.05 ± 0.05	0.41 ± 0.19	0.15
Acetate	54.41 ± 4.06	64.89 ± 9.92	0.75
Propionate	19.61 ± 1.62	21.25 ± 3.43	0.59
iso-Butyrate	0.86 ± 0.37	2.60 ± 0.92	0.17
n-Butyrate	13.54 ± 1.46	15.34 ± 3.68	0.40
iso-Valerate	1.52 ± 0.39	2.71 ± 1.13	0.99
n-Valerate	0.84 ± 0.31	1.65 ± 0.68	0.73
Total organic acid	92.65 ± 6.46	115.63 ± 18.95	0.95
Putrefactive metabolites (µg/g of wet feces)			
Phenol	4.65 ± 0.73	4.78 ± 0.54	0.56
para-Cresol	73.66 ± 15.63	85.57 ± 24.70	0.89
Indole	63.23 ± 13.58	38.95 ± 11.68	0.09
Skatole	6.43 ± 3.81	21.92 ± 14.67	0.87

\*p&lt;0.05.

produce more organic acids than putrefactive metabolites.

The prolonged, high-intensity exercise that endurance runners routinely carry out may have resulted in a form of dysbiosis. For example, the production of reactive oxygen species and inflammatory cytokines associated with gut ischemia-reperfusion may have been caused by this kind of exercise, which may have negatively affected the gut microbiota due to their bactericidal properties.<sup>(1,5,39)</sup> The exercise also likely caused gut injury and malabsorption of nutrients. Nonetheless, the underlying mechanism by which intensive, prolonged exercise affects the gut microbiota modification still remains unclear.

To conclude, in the present study, we showed that female elite endurance runners had gut microbiotas and metabolites profiles that differed from those observed in age-matching female controls. In addition, a higher abundance of *Faecalibacterium* in the gut microbiota of the female elite endurance runners may be involved in the accumulation of succinate. We theorize that a

higher abundance of inflammation-related bacteria and a higher concentration of succinate may have resulted in a form of dysbiosis in the athletes. We suggest that the relationship between the microbiota and the production of bacterial metabolites in the gut of athletes be further evaluated in future studies.

### Author Contributions

WA, TTakagi, YN, and RI conceived this experiment; WA and AK enrolled the participating subjects and collected fecal samples; SM, TK, and RI conducted the experiments; SM, TTsukahara, and RI analyzed data; SM, TTsukahara, and RI wrote the manuscript. All authors have read and approved the final manuscript.

### Conflict of Interest

No potential conflicts of interest were disclosed.

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