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Full Paper

Effect of probiotic *Limosilactobacillus reuteri* on a serum sex-hormone- and metabolic-syndrome-related health evaluation: a single-arm open-label pilot study

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The oral administration of living *Limosilactobacillus reuteri* (formerly *Lactobacillus reuteri*) ATCC PTA 6475 cells has been shown preliminarily to prevent obesity in diet-derived obese mice and to act to ameliorate the decline in serum testosterone in old male mice. Through a clinical trial comprising a single-arm open-label pilot study in which all subjects received the same intervention, the present study aimed to evaluate whether *L. reuteri* ATCC PTA 6475 cells can also ameliorate the decline in serum testosterone levels in senior citizens and explore how the strain changes the intestinal microbiota. The trial was conducted with 10 eligible subjects (aged 50–69) at Hiroshima University from January to April 2024. They were instructed to take two capsules that contained a total of 1.0×10^{10} living lyophilized cells of ATCC PTA 6475 strain every day. After the 12 weeks, although remarkable changes in sex hormones were not observed, significant decreases were observed in body fat percentage, blood pressure, and some inflammation-related parameters. In addition, analysis of the fecal microbiota indicated that intake of ATCC PTA 6475 cells significantly increased the relative abundance of the genera *Butyricimonas*, *Holdemania*, and *Odoribacter*, which have been reported to contribute to the amelioration of obesity phenotypes. In conclusion, although the present study was carried out as a pilot study with only 10 subjects, making a placebo-controlled study necessary in the future, it demonstrates the probiotic potential of the ATCC PTA 6475 strain.

Key words: testosterone, estradiol, lactic acid bacteria, microbiota, clinical trial

INTRODUCTION

It is generally acknowledged that several important sex hormone levels, including testosterone, gradually decrease in men after the age of 40 [1]. Age-related reductions in serum testosterone levels may result from significant decline in the ability of aged Leydig cells in the testicles to respond to luteinizing hormone, which stimulates Leydig cells to produce testosterone [2]. On the other hand, the serum concentrations of luteinizing hormone and another pituitary gonadotropin, follicle-stimulating hormone, which also plays a role in stimulating testosterone secretion, increase with age in men [3]. Thus, the reduced serum testosterone levels may be due to primary gonadal failure rather than disorder in the metabolism of pituitary gonadotropins [4, 5]. The insufficient production of testosterone has been reported to cause not only hypogonadism and hypoactive sexual desire but also an increase in body fat, decrease in bone density, loss of muscle mass, fatigue, and depression; therefore, a reduction in serum testosterone levels has been regarded as a root cause of poor health and the cause of male menopause symptoms [6–10]. In females, although its production level is only 5–10% that in males, testosterone is also synthesized in the ovaries and adrenal glands and plays a necessary role in the maintenance of bone, muscle, sexual desire, and vitality [11]. Insufficient testosterone that comes with aging, as in the case of males, may also result in physical and mental disorders.

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Possible causes of decreased testosterone production in mice and human beings include obesity and a westernized diet, and there may be a complicated relationship between obesity-related disorders and decreased testosterone [12–15]. Recent studies have revealed that the oral administration of live *Limosilactobacillus reuteri* (formerly *Lactobacillus reuteri*) ATCC PTA 6475 cells prevents obesity in diet-induced obese mice [16]. The strain has also been reported to be effective for ameliorating the decline in testosterone levels and producing luxuriant fur in aged male mice [17, 18], which are predicted to be due to improvement of the gut microbiota and the resultant well-balanced intestinal immunity, especially reduction of the inflammation-stimulating cytokine interleukin (IL)-17.

Therefore, in the present study, a clinical trial was conducted as a single-arm open-label pilot study in which all subjects received the same intervention with the aim being to show whether *L. reuteri* ATCC PTA 6475 cells can also maintain youthful serum testosterone levels in senior citizens and explore how the strain changes intestinal microbiota.

MATERIALS AND METHODS

Subjects

Through a series of advertisements, we recruited healthy volunteers between 50 and 69 years of age in Hiroshima, Japan, and its surrounding areas. The inclusion criteria required subjects to be healthy males or females between the ages of 50 and 74. Subjects were excluded if they (1) took medicine for a chronic disease, (2) were predicted by screening to be sick, (3) were premenopausal (female), (4) used supplements or functional foods that may affect sex hormones and the fecal microbiota, or (5) had taken part in other clinical trials within 3 months of the commencement of the present trial. Before the start of the trial, written informed consent was obtained from each subject for the use of their clinical samples and data in research.

Test sample

The test sample in the present study was provided as a capsule containing 5×10^9 living lyophilized cells of *L. reuteri* ATCC PTA 6475 (in 1 capsule), which was produced by BioGaia Japan Inc. (Tokyo, Japan).

Study design

The present clinical study was conducted from January 2024 to April 2024 at Hiroshima University (Hiroshima, Japan) as an open-label, single-arm, non-randomized, single-center trial. After an information session, subjects who met the inclusion criteria were enrolled in the study.

During the 12-week study period, the subjects were instructed to (1) maintain their ordinary exercise and eating habits as much as possible; (2) refrain from donating blood; (3) take two test capsules daily at any time during the day; (4) visit Hiroshima University for a physical examination, biochemical measurements, and urinalysis every 4 weeks; and (5) make a record of the contents of their meals (for 3 days before each examination/measurement), their intake of the test capsules, their health conditions, and their medication, according to the daily dated diary record forms we provided.

In the present study, the primary outcome was the change in total and free testosterone concentrations during the study period. Secondary outcomes included body mass index (BMI), body fat percentage, estimated bone mass, serum estradiol, which is a metabolite of testosterone, and fecal microbiota.

The protocol of the present trial was approved by the Ethics Committee of Hiroshima University (approval no. C2023-0012; date of approval: November 17, 2023) prior to advertisement for subject recruitment, and it was carried out according to the guidelines of the Helsinki Declaration. This study was also registered in the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) under ID UMIN000052825 (date of registration: November 17, 2023).

Physical examination and biochemical measurements

Blood and urine samples were obtained after at least 9 hr of fasting and sent to SRL Inc. (Tokyo, Japan) for clinical biochemical measurements and urinalysis, respectively, every 4 weeks. Body weight, body fat percentage, and estimated bone mass were measured using a BC-118E body composition analyzer (Tanita, Tokyo, Japan), which can calculate body fat percentage and estimated bone mass via multiple regression analysis based on the correlation between the data obtained from bioelectrical impedance analysis (BIA) and dual energy X-ray absorptiometry (DXA) methods [19] using individually measured body weights and bioelectrical impedances. To measure blood pressure, a fully automatic HBP-9020 sphygmomanometer (Omron, Kyoto, Japan) was used, and the data were recorded according to the 2019 Japanese Society of Hypertension Guidelines for the Management of Hypertension (JSH2019) [20]. In accordance with Common Terminology Criteria for Adverse Events version 5.0 (CTCAE v5.0), newly emerged or worsened adverse events that occurred in the subjects after the intervention were evaluated when their grades shifted higher.

Analysis of the fecal microbiota

The fecal microbiota was analyzed by sequencing the V3–V4 region of 16S rRNA-encoding genes. Each fecal sample at weeks 0 and 12 (before and after the intake period) was collected within 3 days of the respective clinical visit. A part of the feces was collected using fecal collection tube FS-0003 (TechnoSuruga Laboratory Co., Ltd., Shizuoka, Japan) filled with 4 mL of RNA*later* Stabilization Solution (Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer's instructions.

Microbial DNA from feces was extracted in accordance with the lytic enzyme–based method [21, 22], with some modifications as previously reported [23]. Each feces sample was homogeneously suspended in the RNA*later* solution, and then undigested food fragments were removed from the suspension by filtration with a 100 μm mesh filter (Sysmex Partec GmbH, Gorlitz, Germany). The microbial pellet was collected from a 2 mL aliquot of the filtrate by centrifugation at 9,000 × g for 15 min at 4°C, and then the pellet was resuspended and washed with 5 mL of phosphate-buffered saline (PBS; pH 7.4) and TE10 buffer (10 mM Tris-HCl, 10 mM Ethylenediaminetetraacetic acid (EDTA), pH 8.0). Each pellet was carefully resuspended in 650 μL of a TE10 buffer containing 23 mg/mL lysozyme (from chicken egg white; Sigma-Aldrich, St. Louis, MO, USA), and the suspension was incubated at 37°C for 1 hr. After the reaction, 40 μL of achromopeptidase solution (Wako Pure Chemical Industries, Ltd.; 50 U/μL stock prepared in a TE10 buffer) was

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added and gently mixed. After further incubation for 30 min, the treated cells were lysed by adding 100 µL of 10% (w/v) sodium dodecyl sulfate (SDS) solution and 10 µL of proteinase K solution (Wako Pure Chemical Industries, Ltd.; 400 U/mL, recombinant) and incubated at 55°C for 1 hr with gentle inversion. Phenol/chloroform/isoamyl alcohol (25/24/1) extraction was performed to denature and remove the protein, and DNA was collected by ethanol precipitation. The obtained DNA sample was further purified by RNase A treatment (Nippon Gene Co., Ltd., Tokyo, Japan), followed by ethanol precipitation.

The microbiota analysis was carried out at Bioengineering Lab. Co., Ltd. (Kanagawa, Japan), using an Illumina MiSeq sequencing platform and MiSeq Reagent Kit v3 (Illumina Inc., San Diego, CA, USA) with a 300 bp read length paired-end method in accordance with the protocol described previously [23]. Briefly, the PCR-amplified V3–V4 region of 16S rDNA fragments was purified and further used as a template for a second PCR reaction using a primer pair with index sequences designed to identify each sample in the analysis system. The amplified fragments were purified and then analyzed with the analytical instrument. The taxonomic assignments of the obtained data were determined using the Quantitative Insights into Microbial Ecology (QIIME) 2.0 pipeline [24].

Statistical analyses

All statistical analyses were performed using SPSS Statistics for Windows, version 17.0 J (SPSS Inc., Chicago, IL, USA). The measured data were analyzed in accordance with the intention-to-treat (ITT) principle [25]. When necessary, 20 multiple-imputed data sets were generated by statistical software, and the resultant analyses were combined to compare each outcome. Wilcoxon's signed rank test was applied for the statistical analyses of the changes in sex hormone profiles and of the relative abundance of each microbe in the fecal microbiota. A paired-samples t-test was applied to compare the differences in other outcomes before and after the intervention.

RESULTS

Subject recruitment and baseline characteristics

The flow diagram in Fig. 1 shows that 20 applicants expressed interest in the present trial, and 15 of them attended the explanatory meeting. After the screening, 10 eligible subjects (aged 50–69) were ultimately confirmed to satisfy the criteria and were subsequently enrolled in the study. Table 1 shows a summary of the subjects' baseline characteristics. During the trial period, one subject dropped out of the study due to family reasons (total attendance rate, 95.0%). The minimum, maximum, and average compliance rates in taking daily capsules, confirmed by the diaries, were 89.3, 100, and 97.5%, respectively.

Effect on sex hormones

Changes in the total and free testosterone concentrations, the primary outcomes, during the 12-week trial period were statistically evaluated (Table 2). Contrary to prior expectations based on previous animal experiments [17, 18], there were few changes in both items after the intervention (p=0.594 and 0.763, respectively). At baseline, two female subjects had total testosterone levels at the lower limit of quantitation (0.03 ng/mL), and one female subject had free testosterone levels at the lower limit of quantitation (0.3 pg/mL). However, after the 12-week intervention, no subjects had values at the lower limit of quantitation for either measure. The results suggest that, although there were no remarkable increments in the hormone, the test sample might have slightly affected the testosterone level.

Although the third quartile (Q3) of estradiol, a metabolite of testosterone measured as a secondary outcome, appeared to have increased at 12 weeks, there was no remarkable change in the median level (p=0.249). In fact, five female subjects consistently had values at the lower limit of quantitation (5 pg/mL) throughout the present trial; however, an abnormal rise (approximately 9 times) was observed in one female subject, resulting in the observed increase of the Q3 value. She had already passed menopause; however, the fluctuation of the sex hormone during the menopausal period might not have completely settled yet.

Effect on secondary physical outcomes

BMI, body fat percentage, and estimated bone mass were also measured and statistically analyzed (Table 3). There was only a slight decrease in BMI after the intervention (p=0.403), whereas the body fat percentage showed an obvious significant decrease from the baseline (p<0.001). There was a small increment in the estimated bone mass, with the change also being statistically significant (p=0.015). The results are consistent with those of previous reports obtained from animal experiments and clinical

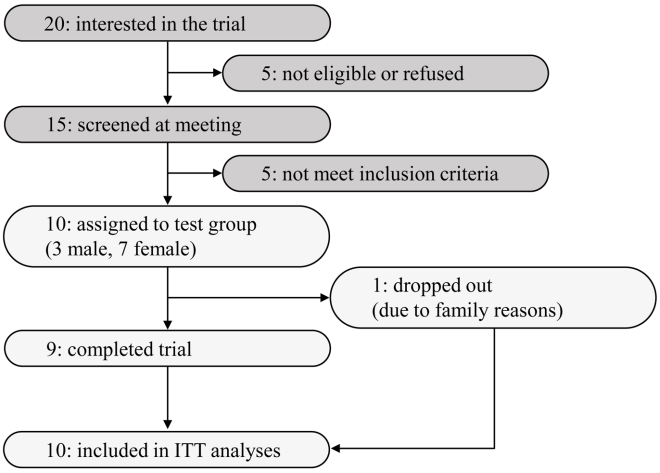

Fig. 1. Flow diagram of the subjects in the present trial.

Table 1. Baseline characteristics of the subjects

Item	Value
Age (years)	57.5 ± 5.4
Male	54.0 ± 4.0 (*n*=3)
Female	59.0 ± 5.4 (*n*=7)
Height (cm)	159.0 ± 7.9
Body weight (kg)	56.3 ± 13.3
BMI (kg/m^2)	22.0 ± 3.8
Estimated bone mass (kg)	2.2 ± 0.5
Systolic blood pressure (mmHg)	117.3 ± 17.8
Diastolic blood pressure (mmHg)	78.3 ± 11.1

Data indicated as mean ± SD. BMI: body mass index.

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trials in diet-induced obese mice and older women with low bone mineral densities [16, 26, 27].

Adverse effect monitoring

For detecting the adverse effects of test sample intake, blood pressure, urinalysis, and hematological and other serum parameters were also analyzed. All of the adverse events observed in the subjects are summarized in accordance with CTCAE v5.0 criteria in Table 4. The observed adverse events were categorized as Grade 1, which is defined as "asymptomatic or mild symptoms, clinical or diagnostic observations only, and intervention not indicated". Some subjects already met the Grade 1 criteria for some items before intake of the test sample. Because no severe adverse events had been reported for test samples in previous clinical studies [17, 18], the test sample was probably irrelevant to the observed adverse events. Among the items monitored throughout the present study, the average serum uric acid level increased significantly (p=0.003) from 5.3 to 5.8 mg/dL, although the change was not abnormal (Table 5). In contrast, there were significant decreases in blood pressure, aspartate aminotransferase (AST), cholinesterase, alkaline phosphatase (ALP), and amylase. Especially in those measurements, both systolic (from 117.3 to 106.8 mmHg, p=0.003) and diastolic (from 78.3 to 72.1 mmHg, p=0.010) blood pressures decreased significantly. Although this study was not designed to target participants with elevated blood pressure levels, the findings strongly suggest that the intake of this strain exerted a substantial influence on blood pressure.

Changes in fecal microbiota

The obtained sequences from the fecal samples were taxonomically annotated as 112 genera. Statistical analysis showed that differences in the relative abundances before

Table 2. Comparison of changes in sex hormones

Items	Baseline	At 12 week	p-value
Total testosterone (ng/mL)			0.594
Median[1]	0.22 (0.07–3.46)	0.25 (0.15–4.08)	
Free testosterone (pg/mL)			0.763
Median	0.75 (0.5–10.0)	0.60 (0.5–8.9)	
Estradiol (pg/mL)			0.249
Median	9.0 (5.0–24.2)	7.4 (5.0–33.7)	

[1]Data indicated as median (interquartile range).
p-values are calculated by Wilcoxon's signed rank test.

Table 3. Comparison of changes in physical secondary outcomes

Items	Baseline	At 12 week	p-value
BMI (kg/m^2)			0.403
Mean[1]	22.0 ± 1.2	21.9 ± 1.2	
Body fat percentage (%)			<0.001***
Mean	26.8 ± 2.5	24.9 ± 2.6	
Estimated bone mass (kg)			0.015*
Mean	2.2 ± 0.17	2.3 ± 0.16	

[1]Data indicated as mean ± SE.
p-values are calculated by paired-samples t-test (*p<0.05; ***p<0.001).

Table 4. Numbers of subjects that showed adverse events after the intervention in the present study

	Number of subjects (n=10)	Note
Blood lactate dehydrogenase increased		
Grade 1	3	
Cholesterol high		
Grade 1	6	Applicable before intervention
Creatinine increased		
Grade 1	2	
Hematuria		
Grade 1	1	
Hypertriglyceridemia		
Grade 1	3	
Hypertension		
Grade 1	4	Applicable before intervention
Hyperuricemia		
Grade 1	1	Applicable before intervention

Table 5. Results for other monitored parameters in the present study

Items	Baseline	At 12 week	p-value
Body weight (kg)			0.263
Mean[1]	56.3 ± 4.2	56.0 ± 4.2	
Systolic blood pressure (mmHg)			0.003**
Mean	117.3 ± 5.6	106.8 ± 5.3	
Diastolic blood pressure (mmHg)			0.010*
Mean	78.3 ± 3.5	72.1 ± 4.2	
Aspartate aminotransferase (U/L)			0.022*
Mean	27.0 ± 3.1	23.7 ± 2.0	
Alanine transaminase (U/L)			0.113
Mean	25.1 ± 7.1	21.1 ± 4.9	
γ-Glutamyl transpeptidase (U/L)			0.365
Mean	28.5 ± 4.2	26.8 ± 4.0	
Lactate dehydrogenase (U/L)			0.302
Mean	214.3 ± 6.8	205.9 ± 8.3	
Cholinesterase (U/L)			0.044*
Mean	345.7 ± 16.0	326.9 ± 13.8	
Alkaline phosphatase (U/L)			0.038*
Mean	79.8 ± 6.1	75.5 ± 6.4	
Amylase (U/L)			<0.001***
Mean	94.2 ± 10.1	86.0 ± 10.3	
Total protein (g/dL)			0.320
Mean	7.3 ± 0.10	7.2 ± 0.11	
Total bilirubin (mg/dL)			0.509
Mean	0.71 ± 0.06	0.77 ± 0.08	
Albumin (g/dL)			0.215
Mean	4.5 ± 0.10	4.6 ± 0.07	
Uric acid (mg/dL)			0.003**
Mean	5.3 ± 0.3	5.8 ± 0.3	
Blood urea nitrogen (mg/dL)			0.559
Mean	16.1 ± 0.9	15.6 ± 1.1	
Creatinine (mg/dL)			0.349
Mean	0.82 ± 0.07	0.83 ± 0.08	
LDL-cholesterol (mg/dL)			0.662
Mean	135.7 ± 7.6	133.3 ± 5.8	
HDL-cholesterol (mg/dL)			0.055
Mean	79.0 ± 7.3	73.2 ± 5.6	
Total cholesterol (mg/dL)			0.141
Mean	238.1 ± 9.9	230.7 ± 8.4	
Triglyceride (mg/dL)			0.940
Mean	98.0 ± 21.4	97.0 ± 22.7	
Fasting blood glucose (mg/dL)			0.287
Mean	105.6 ± 3.9	101.1 ± 3.0	

[1]Data are indicated as mean ± SE.
p-values are calculated by paired-samples t-test (*p<0.05; **p<0.01; ***p<0.001).

and after the intervention wre statistically significant for the *Bifidobacterium*, *Butyricimonas*, *Holdemania*, and *Odoribacter* genera (Fig. 2A). The analysis also revealed that the *Anaerofustis*, *Anaerotruncus*, *Haemophilus*, and *Lactobacillus* genera show remarkable changes in their relative abundances; the changes were not statistical significant but did show favorable statistical trends (p<0.1). Excluding *Bifidobacterium* and *Haemophilus*, their abundances increased after the trial period. A detailed analysis at the annotated species level showed that the respective increments for *L. reuteri* (annotated as the previous taxonomic classification) and *Bacteroides caccae* were statistically significant (p=0.012 and 0.028) and that the decrement for *Haemophilus parainfluenzae*, which is the only annotated species in the *Haemophilus* genus, showed a trend toward significance (p=0.075; Fig. 2B).

The microbiota analysis also revealed that *L. reuteri* was not detected in any feces of the subjects at baseline; however, intake of the test sample undeniably increased its relative abundance. A few changes were observed in other annotated *Lactobacillus* strains (including previous taxonomic classifications)—*Lacticaseibacillus zeae*, *Limosilactobacillus mucosae*, *Lactobacillus delbrueckii*, and *Ligilactobacillus salivarius*—indicating an increase in the *Lactobacillus* genus with a favorable statistical trend (p=0.051; Supplementary Fig. 1). In addition to *L. reuteri*, species level annotation revealed an increase in *B. caccae* after the trial period. *Bacteroides* species *Bacteroides eggerthii*, *Bacteroides fragilis*, *Bacteroides ovatus*, *Bacteroides plebeius*,

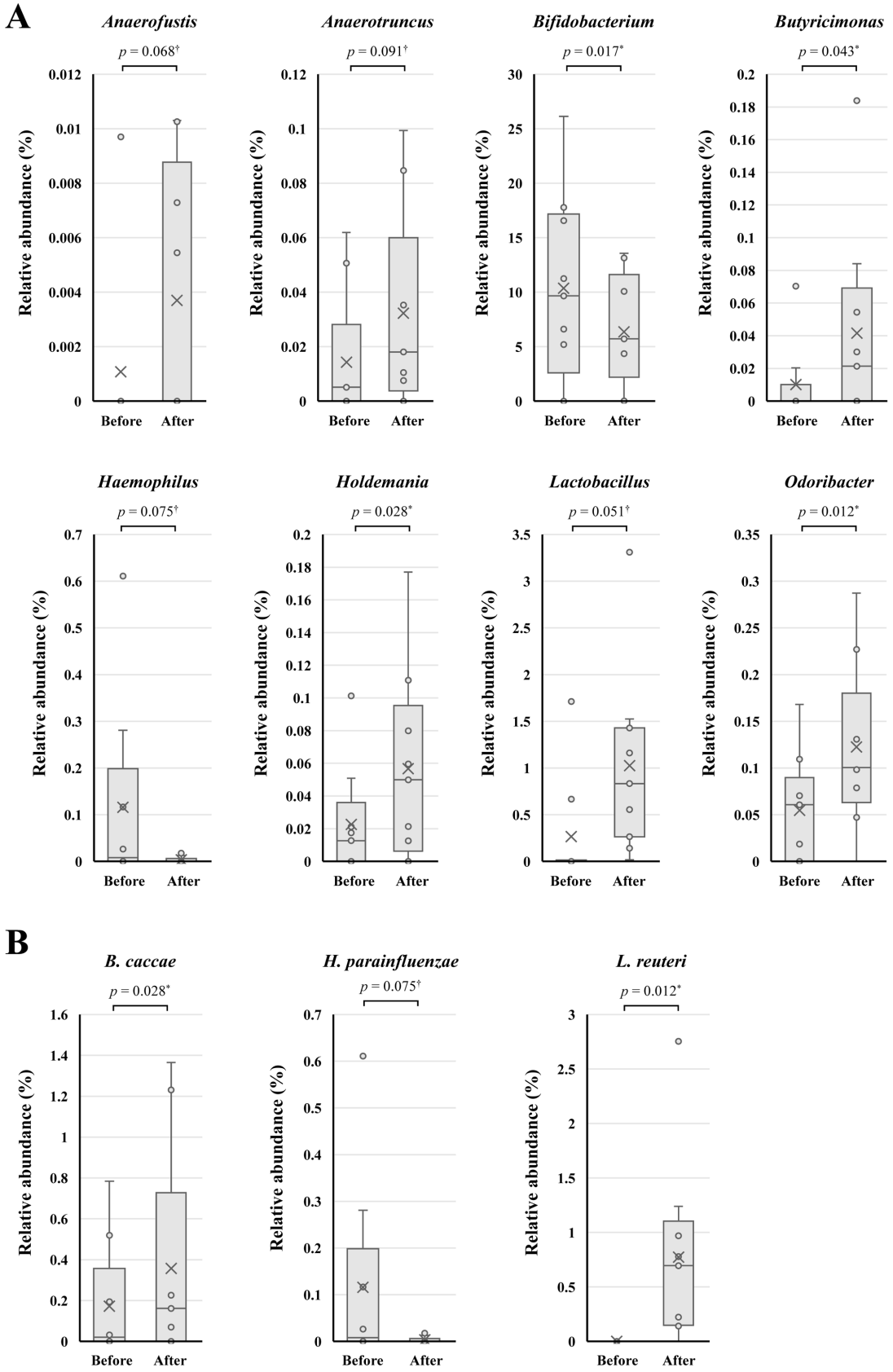

Fig. 2. Statistical analysis of the fecal microbiota levels at the genus (A) and species (B) levels before and after the intake period.

The relative abundances before and after the intake period were compared individually. Data are shown as a box plot with medians (lines inside boxes), means (cross marks), 25–75 percentile (limits of boxes), whiskers indicating the 95% data range, and outliers indicated by circles outside the whiskers. The statistical analyses were performed using Wilcoxon's signed rank test.

†p<0.1. *p<0.05.

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and *Bacteroides uniformis* were detected along with *B. caccae*; however, five other species showed no noticeable changes in their abundances (Supplementary Fig. 2). Different from *L. reuteri*, which contributed the increase in the *Lactobacillus* genus, the abundance of the *Bacteroides* genus at baseline was almost same as that at the end of the intervention period (medians of 9.1% and 10.4%, respectively); the change in *B. caccae* seems to have been distinctive among them.

Only the *Bifidobacterium* genus, which is well known as a representative probiotic, showed a statistically significant decrease in the present analysis, and the change was quite a contrast to the results for the other genera described above, with their desirable correlations with and contributions to health-promoting effects. Decreases in the abundances of the three annotated *Bifidobacterium* species—*Bifidobacterium adolescentis*, *Bifidobacterium bifidum*, and *Bifidobacterium longum*—were undeniably observed; however, no statistical differences were detected in them (Supplementary Fig. 3). Although our phylum-level analysis indicated upward trends in the relative abundances of Bacteroidetes and Proteobacteria, a statistically significant difference was only observed for the decrease in the abundance of Actinobacteria, to which the genus *Bifidobacterium* belongs (Fig. 3, p=0.048).

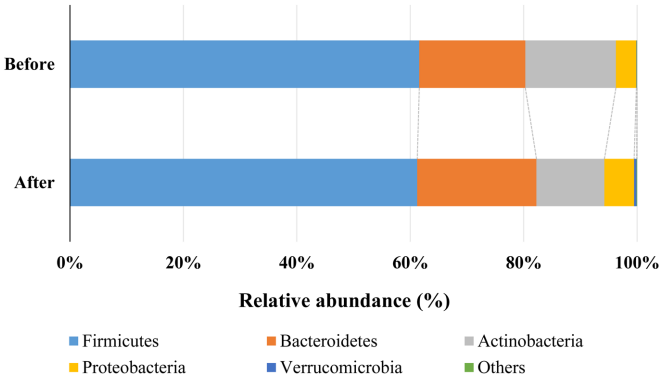

Fig. 3. Differences in the relative profiles of representative phyla before and after the intake period.

DISCUSSION

The present pilot study showed that neither the means nor medians of the total and free testosterone concentrations, which were the primary outcomes, were affected much by intake of the test sample. As a secondary outcome, the decrease in body fat percentage was remarkable and statistically significant. A previous animal experiment using outbred Swiss mice revealed that intake of *L. reuteri* ATCC PTA 6475 cells for 9 months prevented both significant body weight increase and subcutaneous fat accumulation [16]. Different from this report, no significant difference in body weight (also BMI) was observed during the 12-week period in the present study, suggesting that longer intervention is required to confirm those effects.

Our analysis of estimated bone mass as another secondary outcome showed a significant increase as a result of intake of ATCC PTA 6475 cells. This result is well consistent with a previous clinical trial that was performed on older women with low bone mineral densities and used the same capsule [26, 27], indicating that the test sample may be expected to be applied to the prevention of aging-associated osteopenia and osteoporosis. In relation to this, ALP, which was monitored to detect adverse effects of test sample intake, was found to have decreased significantly. There are three isozymes of ALP (tissue-specific intestinal, placental, and germ-cell ALPs) and some isoforms of tissue non-specific ALPs, and each enzyme is expressed in a variety of tissues [28]. Although the isoforms of bone ALP, which are relatively prominent in blood [29–31], were not individually measured, the significant decrease observed in ALP might be due to the increase in estimated bone mass. In other words, it is possible that the favorable maintenance of bone metabolic balance results in the repression of the release of bone ALP caused by osteoblasts.

The genera *Butyricimonas*, *Holdemania*, and *Odoribacter*, which were also found to be statistically significantly increased in the present study, have been reported to show negative correlations with obesity and/or diabetes-related disorders. The *Butyricimonas* species are intestinal bacteria resident in mammals, including humans and rats [32], and it has been clarified that an increased abundance of the *Butyricimonas* genus is linked to a lean phenotype in mice and is also associated with decreased triglycerides, BMI, and HDL in humans [33, 34]. In addition, oral fecal microbiota transplantation using fecal material collected from mice treated with rosuvastatin, which increased the abundance of the *Butyricimonas* genus, resulted in improvement of hyperglycemia [35]. The *Holdemania* genus consists of two species—*Holdemania filiformis* and *Holdemania massiliensis* [36, 37]. It has been reported that the intestinal abundance of the former species in obese people is significantly lower than that in lean people [38]. The latter species was isolated from the fecal samples of females suffering from anorexia nervosa [37], indicating that the two species oppositely correlate with the obesity phenotype. Furthermore, the abundance of *Holdemania* has shown positive correlation with improvement in the blood glucose level during the oral glucose tolerance test [39, 40]. These reports seem to correspond well with results showing decreases in subcutaneous fat and body fat percentage observed in *L. reuteri* ATCC PTA 6475-fed mice [16] and those of the present study.

Although there was no statistical significance in the fasting glucose level, which was measured as neither a primary nor secondary outcome, the serum concentrations of glucose in all but one subject decreased uniformly. It is of interest that alteration of the gut microbiota caused by westernized dietary habits, especially the decreased abundance of *Odoribacter*, was shown to be involved in the pathogenesis of insulin resistance in an epidemiologic study with two cohorts of Japanese men who were of the same race but lived either in Japan or the USA [41]. The genus *Odoribacter* consists of three species, one of which—*Odoribacter laneus*—has been reported to improve the blood glucose level and inflammation in obese mice treated by oral administration [42, 43]. *Odoribacter* can produce butyric acid and sulfobacin B, which have been shown to improve insulin sensitivity and lipopolysaccharide-induced inflammation, respectively [44–46]. Although more well-designed studies with many participants are needed, judging from the above reports, the decrease in fasting glucose observed in the present study may be due, at least partially, to the increase in the abundance of the *Odoribacter* genus.

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In a previous study employing the ATCC PTA 6475 strain [27], a significant increase was reported in several bacterial species—including *Ruminococcus albus*, *Escherichia coli*, *Corynebacterium lipophiloflavum*, *Clostridium acetobutylicum*, *Anaerococcus tetradius*, and various *Lactobacillus* spp.—despite the absence of a placebo group and differences in the study population. In contrast, the present study conducted partial species-level identification (Supplementary Fig. 1) and detected *L. reuteri* as the sole representative of *Lactobacillus* spp., with no observable changes in the other aforementioned taxa. In the study by Li *et al.* [27], phylum-level comparisons revealed minimal changes in their good response group (GR), whereas their poor response group (PR) demonstrated increases in Proteobacteria and Actinobacteria, along with a decrease in Verrucomicrobia. In the present study, a marked reduction in *Bifidobacterium* was observed, which corresponded with a decline in the Actinobacteria phylum (Fig. 3). Although increases in several taxa within the phylum Firmicutes, including *Lactobacillus*, were detected—as illustrated in Fig. 2A—the overall proportion of Firmicutes remained largely unchanged (approximately 61%). In contrast, Bacteroidetes exhibited an increase of approximately 2.5%, and Proteobacteria increased by about 1.5%, collectively accounting for the observed ~4% decrease in Actinobacteria. These cumulative microbial shifts likely underlie the reduction in the *Bifidobacterium* genus. Taken together, these findings suggest that the gut microbiota response to *L. reuteri* administration may vary considerably depending on the demographic and physiological background of the study population. Given the limited sample sizes of each group in the studies by Li *et al.* [27] (GR and PR, individually) and our own study (n=10), further research with larger cohorts is warranted to substantiate these observations.

Both the systolic and diastolic blood pressures were found to statistically decrease. There were no descriptions of effects on blood pressure in previous clinical studies using the same strain [26, 27], making the present study the first report regarding this potential. Although direct investigations into the effects of *L. reuteri* on hypertension remain limited, Xiong *et al.* have evaluated the ameliorating effect of *L. reuteri*, in combination with *Limosilactobacillus johnsonii*, in a hypertensive rat model [47]. Their findings demonstrated that the antihypertensive effects were associated with modulation of the gut microbiota, elevated levels of fecal short-chain fatty acids (SCFAs), and a reduction in pro-inflammatory cytokines, collectively contributing to the improvement in blood pressure. In the present study, analysis of the gut microbiota revealed that an increased relative abundance of *B. caccae*, which is reported to be an early colonizer during formation of the gut microbiota in infancy, plays an important role in the mucosal immune system and is associated with SCFA production and anti-inflammatory properties [48–52]. Some microbiota studies have reported that the abundances of the *Desulfovibrio*, *Klebsiella*, *Parabacteroides*, and *Prevotella* genera and the Streptococcaceae family are positively correlated with hypertension [53–55], whereas no remarkable changes in them were observed in the present study. Although it is not obvious why a mild decrease in blood pressure was observed, it is presumed that improvements in body fat, which has been known to correlate with raised blood pressure [56], blood glucose, and inflammation-related measurements, synergistically resulted in the vasodepressor activity.

In the present study, changes in the total and free testosterone concentrations and obesity-related measurements, which were predicted to be affected by the intake of *L. reuteri* ATCC PTA 6475 cells based on previous animal experiments, were analyzed through a clinical trial. Although the changes in the total and free testosterone concentrations were not significant, the changes in the obesity-related measurements were noticeable and correlated with specific fecal bacterial abundances. Furthermore, although the present study was carried out as a pilot study with only 10 subjects, necessitating a placebo-controlled study in the future, the results support the probiotic potentials of the ATCC PTA 6475 strain.

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CONFLICT OF INTEREST

None.

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