

## MICROBIOLOGY

# *Bacteroides uniformis* and its preferred substrate, $\alpha$ -cyclodextrin, enhance endurance exercise performance in mice and human males

Hiroto Morita<sup>1†</sup>, Chie Kano<sup>1†</sup>, Chiharu Ishii<sup>2</sup>, Noriko Kagata<sup>2</sup>, Takamasa Ishikawa<sup>2</sup>, Akiyoshi Hirayama<sup>2</sup>, Yoshihide Uchiyama<sup>3,4</sup>, Susumu Hara<sup>3,5</sup>, Teppei Nakamura<sup>1</sup>, Shinji Fukuda<sup>2,6,7,8\*</sup>

Although gut microbiota has been linked to exercise, whether alterations in the abundance of specific bacteria improve exercise performance remains ambiguous. In a cross-sectional study involving 25 male long-distance runners, we found a correlation between *Bacteroides uniformis* abundance in feces and the 3000-m race time. In addition, we administered flaxseed lignan or  $\alpha$ -cyclodextrin as a test tablet to healthy, active males who regularly exercised in a randomized, double-blind, placebo-controlled study to increase *B. uniformis* in the gut (UMIN000033748). The results indicated that  $\alpha$ -cyclodextrin supplementation improved human endurance exercise performance. Moreover, *B. uniformis* administration in mice increased swimming time to exhaustion, cecal short-chain fatty acid concentrations, and the gene expression of enzymes associated with gluconeogenesis in the liver while decreasing hepatic glycogen content. These findings indicate that *B. uniformis* enhances endurance exercise performance, which may be mediated by facilitating hepatic endogenous glucose production.

## INTRODUCTION

Although training can improve human exercise performance, other factors can also have an impact. For example, several recent studies have shown that gut microbiota could contribute to exercise performance (1–5). Studies have shown that gut microbes are associated with exercise performance in mice; for example, Hsu *et al.* (6) reported that germ-free mice have a shorter swimming endurance than specific pathogen-free (SPF) mice and *Bacteroides fragilis*-monoassociated mice. In addition, Huang *et al.* (7) showed that inoculation with *Eubacterium rectale* or *Clostridium coccoides* improved the swimming time of gnotobiotic mice. Moreover, mice with disrupted gut microbiota due to multiple antibiotic treatments had a shorter treadmill running time than the control (8). *Veillonella atypica* from the gut microbiome of marathon runners increased treadmill running time in SPF mice by producing propionate from exercise-induced lactate (9). In contrast, other studies have demonstrated that exercise alters the gut microbiome profile in mice (10, 11). Despite the lack of evidence linking gut microbes to human exercise performance, studies have reported that the gut microbiota of athletes are more diverse (12, 13) and have a different composition

than nonathletes (12). These findings suggest that the gut microbiota may play a role in the host's physical performance, and the relationship between the microbiota and exercise appears to be bidirectional. However, there is insufficient evidence to establish a causal relationship between exercise performance and specific gut microbes, and whether other human gut microbes are associated with exercise performance remains unknown.

Therefore, this study aimed to identify additional gut microbes associated with endurance exercise performance and determine whether the identified species can influence exercise performance. To this end, we first conducted a cross-sectional study of Japanese long-distance runners and age- and sex-matched nonathletes and analyzed their gut microbiome, and identified *Bacteroides uniformis*, a dominant bacterial species in the human gut (14), associated with endurance exercise performance (3000-m run time). We then found that the supplementation with  $\alpha$ -cyclodextrin ( $\alpha$ CD) in human males increased gut *B. uniformis* abundance and improved endurance exercise performance and that *B. uniformis* administration in mice enhanced endurance exercise performance, possibly by facilitating endogenous glucose production in the liver during exercise.

## RESULTS

### Gut microbiota profile of Japanese long-distance runners was distinct from that of nonathletes

We conducted a cross-sectional study to identify bacterial groups associated with endurance exercise. Fecal samples were collected from 48 male long-distance runners specializing in ekiden (a type of long-distance relay running) who were members of the university ekiden team [athlete group; age, 20.3  $\pm$  1.0 years (mean  $\pm$  SD); height, 169.9  $\pm$  4.2 cm; body weight, 54.8  $\pm$  3.5 kg; and body mass index (BMI), 19.0  $\pm$  0.9] and 10 males who served as controls

<sup>1</sup>Core Technology Laboratories, Asahi Quality & Innovations Ltd., 1-1-21, Midori, Moriya, Ibaraki 302-0106, Japan. <sup>2</sup>Institute for Advanced Biosciences, Keio University, 246-2 Mizukami, Kakuganji, Tsuruoka-shi, Yamagata 997-0052, Japan. <sup>3</sup>Aoyama Gakuin University Track and Field Club, Aoyama Gakuin University, 4-4-25 Shibuya, Shibuya-ku, Tokyo 150-8366, Japan. <sup>4</sup>School of International Politics, Economics and Communication, Aoyama Gakuin University, 4-4-25 Shibuya, Shibuya-ku, Tokyo 150-8366, Japan. <sup>5</sup>School of Global Studies and Collaboration, Aoyama Gakuin University, 4-4-25 Shibuya, Shibuya-ku, Tokyo 150-8366, Japan. <sup>6</sup>Gut Environmental Design Group, Kanagawa Institute of Industrial Science and Technology, 3-25-13 Tonomachi, Kawasaki-ku, Kawasaki, Kanagawa 210-0821, Japan. <sup>7</sup>Transborder Medical Research Center, University of Tsukuba, 1-1-1 Tennodai, Tsukuba-shi, Ibaraki 305-8575, Japan. <sup>8</sup>Laboratory for Regenerative Microbiology, Juntendo University Graduate School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan.

\*Corresponding author. Email: sfukuda@sfc.keio.ac.jp

†These authors contributed equally to this work.

(nonathlete group; age,  $22.6 \pm 0.7$  years; height,  $169.7 \pm 7.6$  cm; body weight,  $59.5 \pm 8.0$  kg; and BMI  $20.6 \pm 2.2$ ).

DNA was extracted from the feces, and the V4 region of the bacterial 16S ribosomal RNA (rRNA)-encoding gene was sequenced using the MiSeq platform to generate a gut microbiota profile. Five participants in the athlete group and two in the nonathlete group were excluded from the sequencing process because polymerase chain reaction (PCR) failed to amplify the target region. This failure could be attributed to PCR inhibitors, such as polysaccharides (15). Ultimately, 6,654,772 sequence reads were analyzed. The mean, minimum, and maximum of the reads per sample were 130,485, 46,053, and 294,588, respectively. The Chao1 estimate ( $P = 0.001$ ), Shannon index ( $P = 0.005$ ), and phylogenetic distance (PD) of the whole tree ( $P = 0.005$ ) revealed that the gut microbiota of the athlete group had significantly higher  $\alpha$ -diversity than that of the nonathlete group (Fig. 1A). In addition,  $\beta$ -diversity analysis based on weighted and unweighted UniFrac distances showed that the microbiota profiles of the two groups were significantly different ( $P = 0.018$  and  $P = 0.001$ , respectively; Fig. 1B). A linear discriminant analysis effect size (LEfSe) analysis demonstrated that the variations in microbiota profiles of the two groups was primarily due to the following nine genera: *Bacteroides*, *Prevotella*, *Lachnospira*, *Sutterella*, an unknown genus of the family Gemellaceae, *Granulicatella*, *Escherichia*, *Citrullus*, and an unknown genus of the order Streptophyta (Fig. 1C). Among these genera, the relative abundance of the following six genera showed significant differences between the two groups: *Bacteroides* ( $P = 0.014$ ; Fig. 1D), *Lachnospira*, and *Sutterella* were highly abundant in the athlete group ( $P = 0.021$  and  $P = 0.021$ ; fig. S1A), whereas *Escherichia* and two unknown genera of Gemellaceae and Streptophyta were abundant in the nonathlete group ( $P = 0.002$ ,  $P = 0.001$ , and  $P = 0.008$ , respectively; fig. S1A).

To determine whether the gut microbiota is associated with endurance exercise performance in long-distance runners, we analyzed the correlations of the relative abundances of the nine genera identified in the LEfSe analysis with exercise-related blood parameters and the 3000-m race time, measured during the same period as that in which the fecal sample collection; however, four of the nine genera (*Citrullus*, unknown genus of the family Gemellaceae, *Granulicatella*, and unknown genus of the order Streptophyta) were excluded because these were detected only in a few participants in the athlete group (fig. S1A). Although all participants provided blood samples, 23 of 48 athletes refused the measurement of their 3000-m race time. In these analyses, only *Bacteroides* showed a significant negative correlation with the 3000-m race time [Pearson correlation coefficient ( $r$ ) =  $-0.441$ ,  $P = 0.028$ ; Fig. 1E and fig. S1B]. The overall trend was a negative correlation between *Lachnospira* and blood parameters; however, the other genera exhibited no patterns (fig. S1C). These findings indicated that the genus *Bacteroides* could be associated with endurance exercise performance.

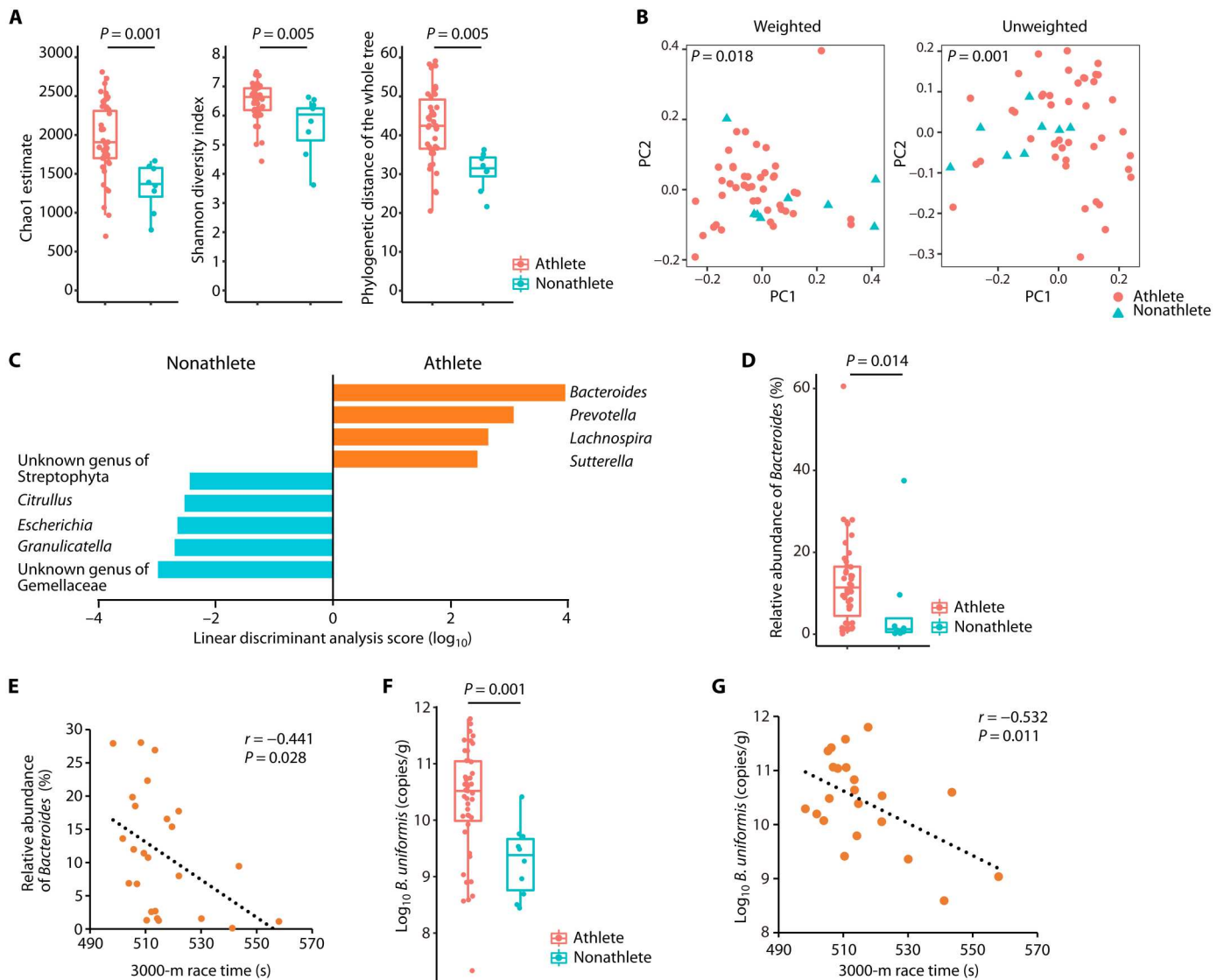
Next, we used species-specific quantitative PCR (qPCR) to determine the abundance of 13 commonly detected *Bacteroides* species in human feces by measuring the copy numbers of their 16S rRNA-encoding gene. *Bacteroides coprocola*, *Bacteroides coprophilus*, *Bacteroides dorei*, *Bacteroides plebeius*, and *Bacteroides vulgatus* were given the new genus name *Phocaeicola* in 2020 (16); however, the Greengenes database used for the gut microbiota analysis in this study does not include the new genus *Phocaeicola*. Thus, the

abundances of these five species are included in the abundance of *Bacteroides* in Fig. 1 (D and E). The qPCR results showed that the abundance of *B. uniformis* (Fig. 1F), *Bacteroides caccae*, *Phocaeicola dorei*, *Bacteroides eggerthii*, *Bacteroides thetaiotaomicron*, and *Phocaeicola vulgatus* (fig. S2A) was significantly higher in the athlete group than that in the nonathlete group ( $P = 0.001$ ,  $P = 0.036$ ,  $P = 0.009$ ,  $P = 0.005$ ,  $P = 0.006$ , and  $P = 0.037$ , respectively). Furthermore, among these six bacterial species, only *B. uniformis* exhibited a significant negative correlation between abundance and 3000-m race time ( $r = -0.532$ ,  $P = 0.011$ ; Fig. 1G and fig. S2B). These results suggested that *B. uniformis* could be associated with endurance exercise performance.

### $\alpha$ CD supplementation increased *B. uniformis* abundance and human male exercise performance

Although *B. uniformis* administration in mice or rats has no adverse outcomes (17, 18), the safety implications of human ingestion have not been explored. Therefore, to investigate whether increasing *B. uniformis* abundance in the human gut affects exercise performance, we needed to increase its abundance using other means. Thus, we sought to identify foods containing substances previously shown to specifically favor the proliferation of *B. uniformis*. Although secoisolariciresinol diglucoside (SDG) is not known to selectively favor the proliferation of *B. uniformis* in the gut, the microbe expresses a gene encoding  $\beta$ -glucosidase, which releases glucose from SDG via hydrolysis (19, 20). Therefore, we tested whether supplementing with flaxseed lignans (FL), which are particularly rich in SDG (21), would affect *B. uniformis* abundance in the gut and human endurance exercise performance. To improve the solubility of SDGs in water, the FL used in this experiment contained approximately 40% (w/w)  $\alpha$ CD, a cyclic oligosaccharide composed of six glucose subunits.  $\alpha$ CD reportedly alters the gut microbiota in mice fed a high-fat diet (22); therefore, we also examined its effects in humans.

We conducted a randomized, double-blind, placebo-controlled, parallel-group human study to test whether FL or  $\alpha$ CD increased intestinal *B. uniformis* abundance and influenced endurance exercise performance. Thirty-six Japanese males aged 20 to 49 years, who exercised regularly (once or twice per week), were randomly assigned to three different groups using age and maximum oxygen consumption ( $\dot{V}O_{2\max}$ ; the maximum capacity of the body to transport and use oxygen during physical activity), which is one of the factors related to endurance exercise performance (23) and was measured 4 weeks before supplementation ( $-4$  weeks), as stratifying factors (Fig. 2A). At the beginning of the study, no significant differences in age, weight, BMI, heart rate, or  $\dot{V}O_{2\max}$  were detected (table S1). For 9 weeks, the three groups were administered a placebo (placebo group), FL (200 mg/day, FL group), or  $\alpha$ CD (200 mg/day,  $\alpha$ CD group) as a supplement. The energy provided by each supplement per daily dose was 3.02, 3.04, and 2.96 kcal for the placebo, FL, and  $\alpha$ CD groups, respectively. The assessments and a schedule of the visits at  $-4$ , 0, 4, 8, and 9 weeks are shown in Fig. 2B. All exercise tests were completed by all participants who attended each visit. One, two, and two participants in the placebo, FL, and  $\alpha$ CD groups, respectively, dropped out of the study for personal reasons. Thus, the analysis ultimately included data from 11, 10, and 10 participants in the placebo, FL, and  $\alpha$ CD groups, respectively. The supplement consumption rates in the placebo, FL, and  $\alpha$ CD groups were  $99.6 \pm 1.0$ ,  $97.1 \pm 4.6$ , and  $97.8 \pm 3.0\%$  (mean  $\pm$  SD),



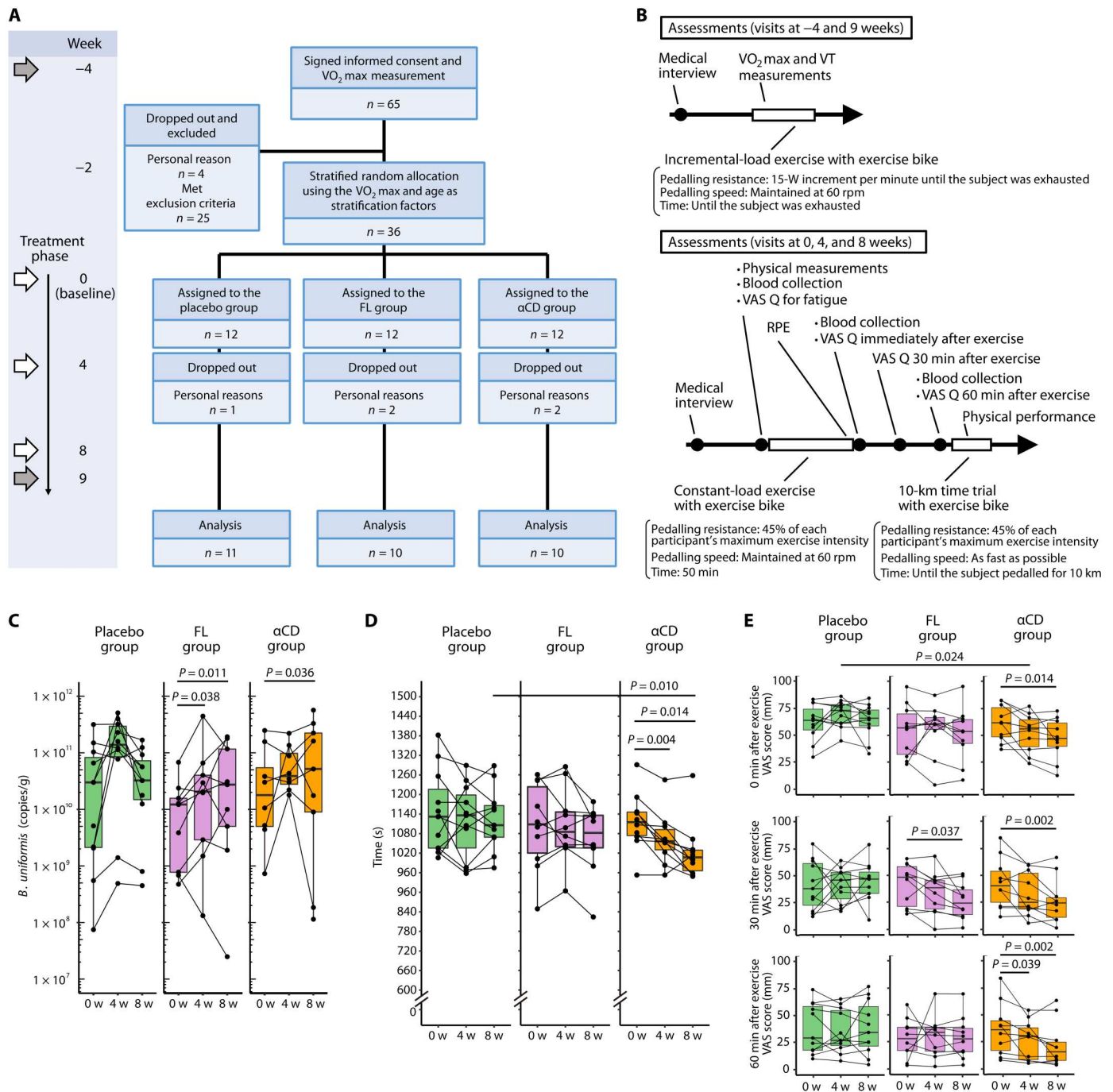
**Fig. 1. *Bacteroides* abundance was higher in long-distance runners and correlated with the 3000-m race time.** (A) Gut microbiota  $\alpha$ -diversity of long-distance male runners (athletes) and nonathlete male controls (nonathletes). The statistical significance of the differences between groups was analyzed using a two-sample *t* test with Monte Carlo permutations. (B) Fecal microbiota profiles of athletes (red circles) and nonathletes (blue triangles). Principal coordinate analysis plots using weighted or unweighted UniFrac distances are shown. The statistical significance of the differences between groups was analyzed using permutational multivariate analysis of variance (PERMANOVA). (C) LefSe analysis was performed to isolate genera with  $|\text{Linear discriminant analysis score}| > 2$ . (D) Relative abundance of the genus *Bacteroides* in the athlete and nonathlete groups, determined by 16S rRNA-encoding gene amplicon sequencing and compared using the Mann-Whitney *U* test. (E) Scatterplot of the 3000-m race time and the relative abundance of *Bacteroides* in the athlete group. Individual participants are represented as circles. (F) *B. uniformis* abundance in feces collected from the athlete and nonathlete groups, determined by 16S rRNA-encoding gene copy numbers using *B. uniformis*-specific qPCR. The Mann-Whitney *U* test was used to compare the groups. (G) Scatterplot of the 3000-m race time and *B. uniformis* abundance in the athlete group. Individual participants are represented as circles. The distribution of values within each group (A, D, and F) is illustrated by a box-and-whisker plot. (A to D) Athlete group,  $n = 43$ ; nonathlete group,  $n = 8$ . (F) Athlete group,  $n = 48$ ; nonathlete group,  $n = 10$ . (E and G) Athlete group,  $n = 25$ . Twenty-three of 48 athletes refused to participate in the trial to measure the race time. All statistical tests were two-tailed. For correlations, the dotted line shows the regression line.

respectively, and the supplements did not cause any adverse events during the experimental period.

To investigate whether FL or  $\alpha$ CD increase the abundance of *B. uniformis* in the human gut, we extracted DNA from fecal samples and performed *B. uniformis*-specific qPCR. The qPCR analysis revealed that after 4 and 8 weeks of supplementation, neither the FL nor the  $\alpha$ CD groups exhibited a significant increase in *B. uniformis*

abundance compared with the placebo group. However, there was a significant increase in *B. uniformis* abundance from baseline at 4 and 8 weeks ( $P = 0.038$  and  $0.011$ , respectively) in the FL group and 8 weeks ( $P = 0.036$ ) in the  $\alpha$ CD group. In the placebo group, the increase in *B. uniformis* abundance at 4 and 8 weeks was not significant (Fig. 2C). Change in *B. uniformis* abundance, between 0 and 8 weeks (8-week abundance minus baseline abundance),





**Fig. 2.  $\alpha$ -Cyclodextrin affected *B. uniformis* abundance, endurance exercise performance, and postexercise fatigue, as demonstrated in a randomized, double-blind, placebo-controlled, parallel-group study.** (A) The randomized, double-blind, placebo-controlled, parallel-group study protocol. The  $\dot{V}O_2$ max was evaluated at the weeks indicated by gray arrows (–4 and 9 weeks). Participants who met the inclusion criteria and did not meet the exclusion criteria were randomly allocated to the placebo, FL, or  $\alpha$ CD groups. At the weeks indicated with white arrows (0, 4, and 8 weeks), we conducted a VAS questionnaire (VAS Q) related to fatigue before and after a 50-min constant-load exercise session, assessed the RPE, measured the time needed to pedal 10 km on an exercise bike, and collected blood and fecal samples. (B) Assessments performed at each visit in chronological order. (C) qPCR analysis of *B. uniformis* abundance in feces from each group at baseline (0 weeks) and after 4 and 8 weeks of supplementation. (D) Time required to pedal 10 km on an exercise bike. (E) Results of the VAS Q on fatigue conducted 0, 30, and 60 min after a 50-min constant-load biking exercise session. Fatigue levels measured by the VAS range from 0, indicating “not feel fatigued at all,” to 100, indicating “feel extreme fatigue.” The distribution of values within each group (C to E) is illustrated by a box-and-whisker plot. Consecutive measurements of the same individuals (dots) are connected with lines. The Mann-Whitney *U* test (placebo group versus FL or  $\alpha$ CD group) or Wilcoxon signed-rank test (0 weeks versus 4 or 8 weeks) were used to determine the statistical significance of the different analyses. All statistical tests were two-tailed.

was  $3.74 \times 10^8$  ( $-2.56 \times 10^{10}$  to  $3.76 \times 10^{10}$ ) [median (interquartile range)],  $1.46 \times 10^{10}$  ( $1.15 \times 10^9$  to  $4.84 \times 10^{10}$ ), and  $3.99 \times 10^{10}$  ( $9.73 \times 10^9$  to  $9.48 \times 10^{10}$ ) copies/g in the placebo, FL, and  $\alpha$ CD groups, respectively. These alterations did not differ significantly between the placebo and FL or  $\alpha$ CD groups. Despite these findings, neither the FL nor the  $\alpha$ CD group showed significant differences in the  $\alpha$ - or  $\beta$ -diversity of the gut microbiota compared with the placebo group (fig. S3).

We measured the 10-km biking time to verify the effects of FL and  $\alpha$ CD on exercise performance. No significant differences were observed in either of the placebo or FL groups at any time points; however, after 4 and 8 weeks of supplementation, the completion times of the  $\alpha$ CD group were significantly shorter than those at baseline ( $P = 0.004$  and  $P = 0.014$ ); at 8 weeks, the completion times were significantly shorter than those of the placebo group ( $P = 0.010$ ), which suggests that  $\alpha$ CD improved endurance exercise performance (Fig. 2D).

Improving endurance exercise performance may have the side benefit of reducing subjective fatigue due to endurance exercise. To confirm this benefit, we measured an individual's fatigue after a 50-min constant-load bike exercise using a visual analog scale (VAS) questionnaire for fatigue and an individual's subjective perception of effort during the exercise using Borg's ratings of perceived exertion (RPE) scale (24). At 0, 30, and 60 min after

exercise, the VAS scores for fatigue in the placebo group did not differ significantly from baseline (Fig. 2E). For the FL group, the VAS score at 0 min immediately after the exercise was not significantly affected throughout the study, and no significant differences compared with the placebo group were observed at any of the time points. In contrast, 30 min after the exercise, the VAS score of the FL group after 8 weeks of supplementation was significantly lower than that at baseline ( $P = 0.037$ ; Fig. 2E). For the  $\alpha$ CD group, significantly lower VAS scores at 0, 30, and 60 min after exercise were found after 8 weeks of supplementation than those at baseline ( $P = 0.014$ ,  $P = 0.002$ , and  $P = 0.002$ , respectively). In addition, at week 4, the  $\alpha$ CD group had a significantly lower VAS score at 0 min than the placebo group ( $P = 0.024$ ; Fig. 2E). For the  $\alpha$ CD group, the RPE score after 50 min of constant road exercise at the 8-week visit was significantly lower than that at baseline ( $P = 0.004$ ), but the same result was also observed in the placebo group ( $P = 0.020$ ; fig. S4).

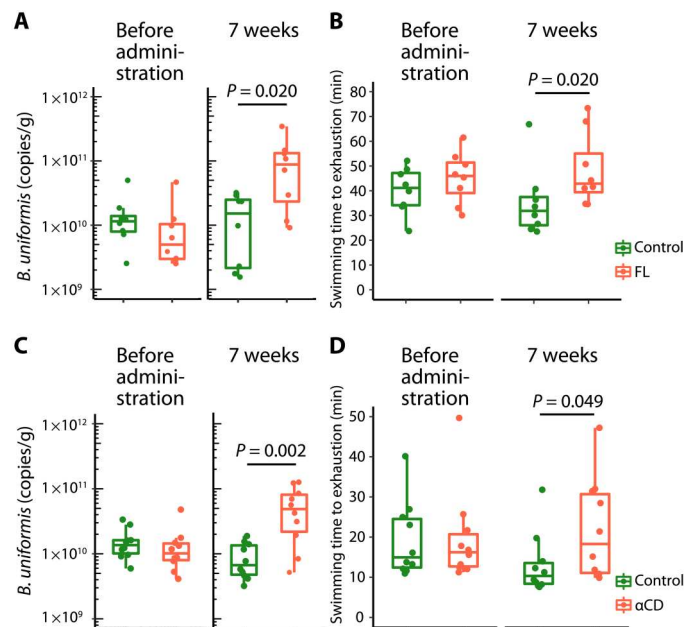
To explore the potential mechanisms underlying the improvements in endurance exercise performance, we measured the  $\dot{V}O_2$ max and ventilatory threshold (VT) as cardiopulmonary function, muscle mass, blood parameters, and concentrations of 30 amino acids in the blood. However, there were no notable changes in these parameters that led to an understanding of the potential mechanisms in humans (figs. S5 to S8).

To summarize the findings of the randomized, double-blind, placebo-controlled, parallel-group study, we found that supplementing with FL or  $\alpha$ CD for 8 weeks increased the *B. uniformis* abundance compared with its abundance before supplementation and that  $\alpha$ CD improved endurance exercise performance.

### Administration of $\alpha$ CD increased *B. uniformis* abundance in the murine intestines and improved endurance exercise performance

To confirm the effects of FL and  $\alpha$ CD on the host, we examined whether supplementation with these substances altered the *B. uniformis* abundance in the gut and the endurance exercise performance in mice. Male C57BL/6J SPF mice (aged 8 weeks) were acclimated to swimming for 1 week and divided into two groups based on each mouse's swimming time to exhaustion. The mice were fed either a standard CE-2 diet (control group) or a CE-2 diet containing 5% (w/w) FL (FL group) ad libitum for 7 weeks, and each group underwent a weekly swimming endurance test in a six-lane, flowing water pool (flow rate program: 12 liter/min for 5 min, 13 liter/min for 5 min, and 14 liter/min until exhaustion; water temperature:  $34^\circ \pm 0.5^\circ\text{C}$ ). After 7 weeks, the FL group had a significantly higher fecal *B. uniformis* abundance as determined by qPCR and higher relative abundance of the genus *Bacteroides* as determined by 16S rRNA-encoding gene sequencing than the control group ( $P = 0.020$  and  $P < 0.001$ ; Fig. 3A and fig. S9A). LEfSe analysis results and gut microbiota compositions at the genus level are shown in fig. S9 (B and C). Furthermore, at the end point, the FL group had a significantly longer swimming time to exhaustion than the control group ( $P = 0.020$ ; Fig. 3B).

The effects of  $\alpha$ CD were also investigated by feeding mice a diet containing 5% (w/w)  $\alpha$ CD ( $\alpha$ CD group) for 7 weeks. A similar significant increase in the fecal *B. uniformis* abundance and a longer swimming time to exhaustion was noted at 7 weeks in the  $\alpha$ CD group compared with the control group ( $P = 0.002$  and  $P = 0.049$ , respectively; Fig. 3, C and D). The relative abundance of *Bacteroides* in feces, LEfSe analysis results, and the genus-level composition of



**Fig. 3. FL and  $\alpha$ CD increased the *B. uniformis* abundance in the murine intestine and prolonged the swimming time to exhaustion.** The mice in the FL or  $\alpha$ CD groups were fed a standard diet containing 5% (w/w) FL or  $\alpha$ CD, respectively, and subjected to a weekly swimming endurance test ( $n = 8$  for the FL group and associated control group;  $n = 10$  for the  $\alpha$ CD group and associated control group). (A and C) Box-and-whisker plots of *B. uniformis* abundance in feces from each group before administration and at the end point (7 weeks) based on the 16S rRNA-encoding gene copy numbers, calculated using *B. uniformis*-specific qPCR. (B and D) Box-and-whisker plots of the swimming time to exhaustion for each group before administration and at the end point (7 weeks). The statistical significance of the differences between groups was analyzed using two-tailed Mann-Whitney *U* tests.

the gut microbiota are shown in fig. S9 (D to F). The LEfSe analysis revealed that only the relative abundance of *Bacteroides* was increased in the  $\alpha$ CD group compared with the control group.

According to these findings, both FL and  $\alpha$ CD increase *B. uniformis* abundance in the murine intestine and improve mouse endurance exercise ability. In particular,  $\alpha$ CD specifically increases the abundance of *Bacteroides* compared with FL.

### ***B. uniformis* administration improved endurance exercise performance in mice**

The results from the human and mouse experiments indicated that *B. uniformis* may improve endurance exercise performance. Thus, we subsequently determined whether *B. uniformis* administration could improve endurance exercise performance in mice. First, we evaluated the effect of an oral administration of the *B. uniformis* on the swimming endurance of SPF mice. For this experiment, 8-week-old male C57BL/6J mice were acclimated to swimming and grouped in the same way as in the experiment used to generate the data shown in Fig. 3. For 4 weeks, both groups received either phosphate-buffered saline (PBS) or *B. uniformis* type strain [ $2 \times 10^8$  colony-forming units (CFU)/day] orally (Fig. 4A). Under the same conditions as the experiment depicted in Fig. 3, a weekly swimming endurance test was performed. At 3 and 4 weeks, the *B. uniformis*-treated mice swam for a significantly longer duration than the PBS-treated control group ( $P = 0.021$  and  $P = 0.014$ , respectively; Fig. 4B). Next, we used *P. dorei*, which, following *B. uniformis*, had the second-highest abundance among the *Bacteroides* genus in the feces of the athlete group, whose abundance was significantly higher than that of the nonathlete group (fig. S2A). However, the swimming time to exhaustion of *P. dorei* type strain-administered SPF mice ( $2 \times 10^8$  CFU/day) showed no significant differences to PBS-administered mice at any time (Fig. 4C). These results suggest that *B. uniformis* administration improves endurance exercise performance in mice.

### ***B. uniformis* administration and periodic exercise altered the intestinal short-chain fatty acid and hepatic glycogen concentrations in mice**

We analyzed the cecal short-chain fatty acid (SCFA) concentrations, hepatic gene expression patterns, hepatic glycogen content, muscle metabolite concentrations, and muscle glycogen content of the mice administered *B. uniformis* and subjected to exercise to explore the underlying biological phenomena. In this experiment, the mice were forced to swim for 15 min once a week (Fig. 5A) but under milder conditions (flow rate program: 12 liter/min for 5 min and 13 liter/min for 10 min; water temperature:  $34^\circ \pm 0.5^\circ\text{C}$ ) than those used in the swimming endurance test to elicit a consistent response to exercise. Immediately following the swimming exercise in week 10, the mice were euthanized, and their cecal content, liver, and quadriceps femoris were collected.

Gas chromatography measurements of cecal SCFA concentrations revealed that the concentrations of acetate, propionate, butyrate, and valerate in the PBS-administered mice with exercise (PBStr<sup>+</sup>) were significantly lower than those in the PBS-administered mice without exercise (PBStr<sup>-</sup>) ( $P < 0.001$ ,  $P = 0.003$ ,  $P = 0.005$ , and  $P = 0.047$ , respectively; Fig. 5B). However, the concentrations of these four SCFAs were comparable in the *B. uniformis*-administered mice with (BUtra<sup>+</sup>) and without exercise (BUtra<sup>-</sup>), and the acetate and propionate concentrations were significantly higher

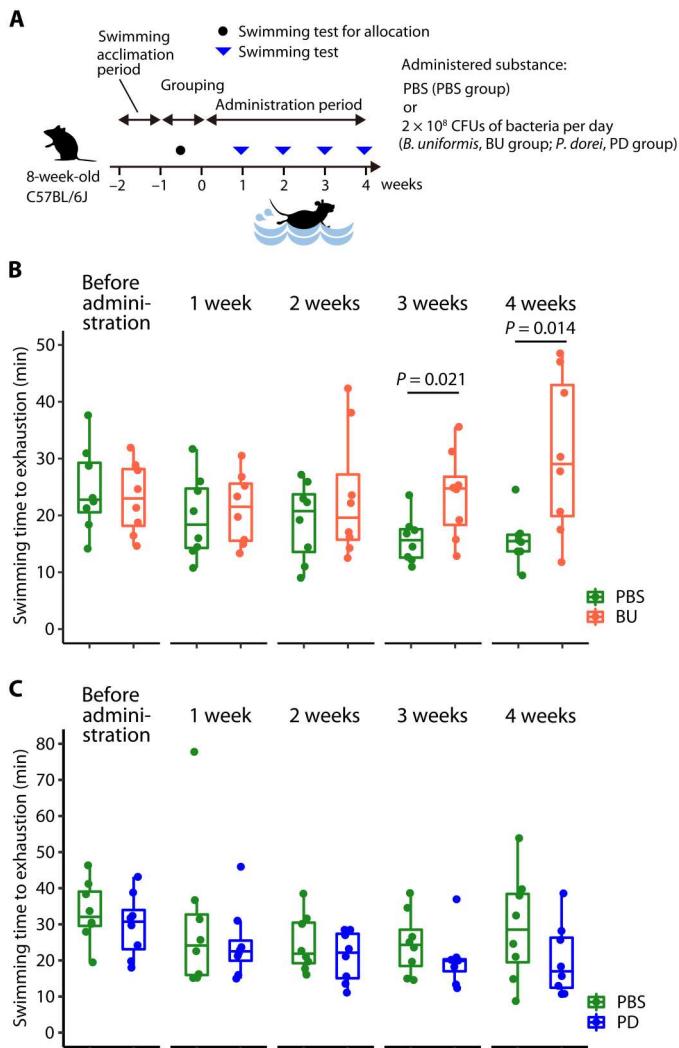
in the BUtra<sup>+</sup> mice than in the PBStr<sup>+</sup> mice ( $P < 0.001$  and  $P = 0.004$ , respectively; Fig. 5B). The isobutyrate and isovalerate concentrations showed different patterns from the four SCFAs. The two SCFA concentrations were similar in PBStr<sup>+</sup> and PBStr<sup>-</sup> and higher in BUtra<sup>+</sup> mice than in BUtra<sup>-</sup> mice ( $P = 0.014$  and  $P = 0.006$ , respectively). These results suggest that exercise reduces the acetate, propionate, butyrate, and valerate concentrations in the cecum, while *B. uniformis* administration prevents this exercise-induced decrease.

During fasting and exercise, liver glycogen is broken down into glucose and used to restore blood glucose levels. Thus, we assessed the hepatic glycogen content of mice in the four groups. The BUtra<sup>+</sup> group, compared with the other three groups, had significantly lower levels of hepatic glycogen ( $P < 0.001$ ,  $P = 0.002$ , and  $P < 0.001$ , respectively; Fig. 5C).

Next, we investigated the hepatic gene expression patterns by performing an RNA sequencing (RNA-seq) analysis of mRNAs isolated from the mice livers in four groups and then conducting a gene ontology (GO) analysis of differentially expressed genes with a  $q$  value of  $<0.05$ . The PBStr<sup>-</sup> versus PBStr<sup>+</sup> and BUtra<sup>-</sup> versus BUtra<sup>+</sup> comparisons identified 91 and 603 differentially expressed genes, respectively. Consistent with prior findings, a GO analysis comparing the PBStr<sup>-</sup> and PBStr<sup>+</sup> groups revealed enrichment in the rhythmic process and circadian rhythm (Fig. 5D) (25, 26). However, the comparison of the BUtra<sup>-</sup> and BUtra<sup>+</sup> groups indicated enrichment of lipid metabolism and gluconeogenesis in addition to GO terms related to the circadian rhythm (Fig. 5E). Moreover, the mRNA levels of *Cpt1a* (GO term: lipid metabolism) and *Pck1* (GO term: gluconeogenesis) measured by reverse transcription qPCR in the livers of the BUtra<sup>+</sup> mice were significantly higher than those in the livers of the BUtra<sup>-</sup> mice ( $P = 0.033$  and  $P = 0.049$ , respectively; Fig. 5F). *Cpt1a* encodes carnitine palmitoyl transferase 1a, which transfers long-chain fatty acids into mitochondria for  $\beta$ -oxidation (27). *Pck1* encodes phosphoenolpyruvate carboxykinase, which catalyzes the reversible conversion of oxaloacetate to phosphoenolpyruvate for gluconeogenesis (28).

To determine whether *B. uniformis* administration and swimming exercise alters muscle metabolism related to energy acquisition, specifically the adenosine triphosphate-creatine phosphate (ATP-CP) system, the tricarboxylic acid (TCA) cycle, glycolysis, and fatty acid  $\beta$ -oxidation, we subsequently performed a capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS)-based metabolome analysis of quadriceps femoris samples from the four groups. However, no significant differences in the muscle metabolites related to energy acquisition were found among the four groups (fig. S10A). Furthermore, there was no significant difference in the transcript levels of *Pgc-1a*, a regulator involved in energy metabolism, or four genes related to glucose metabolism (*Hk1*, encoding hexokinase 1; *Hk2*, encoding hexokinase 2; *Pygm*, encoding muscle glycogen phosphorylase; and *Glut4*, encoding solute carrier family 2, member 4) in the quadriceps femoris muscles of the groups (fig. S10B). Moreover, the quadriceps femoris glycogen content showed no significant differences among the four groups (fig. S10C). These results indicate that the *B. uniformis* administration and weekly exercise performed in this experiment did not affect the ATP-CP system, TCA cycle, glycolysis, fatty acid  $\beta$ -oxidation, or glycogen content in the quadriceps femoris.





**Fig. 4. *B. uniformis* administration improved endurance exercise performance in mice.** (A) Endurance exercise test schedule. The mice were administered PBS (PBS group), *B. uniformis* ( $2 \times 10^8$  CFU/day) (BU group), or *P. dorei* ( $2 \times 10^8$  CFU/day) (PD group) for 4 weeks and tested weekly for swimming endurance. (B and C) Swimming time to exhaustion of PBS-administered ( $n = 8$ ), *B. uniformis*-administered ( $n = 8$ ) (B), and *P. dorei*-administered ( $n = 8$ ) (C) mice. The box-and-whisker plot illustrates the distribution of values within each group. The statistical significance of the differences between groups was analyzed using two-tailed Mann-Whitney *U* tests.

### Fecal SCFA levels were higher, and hepatic glycogen levels were lower than those of germ-free mice in exercised *B. uniformis*-monoassociated mice

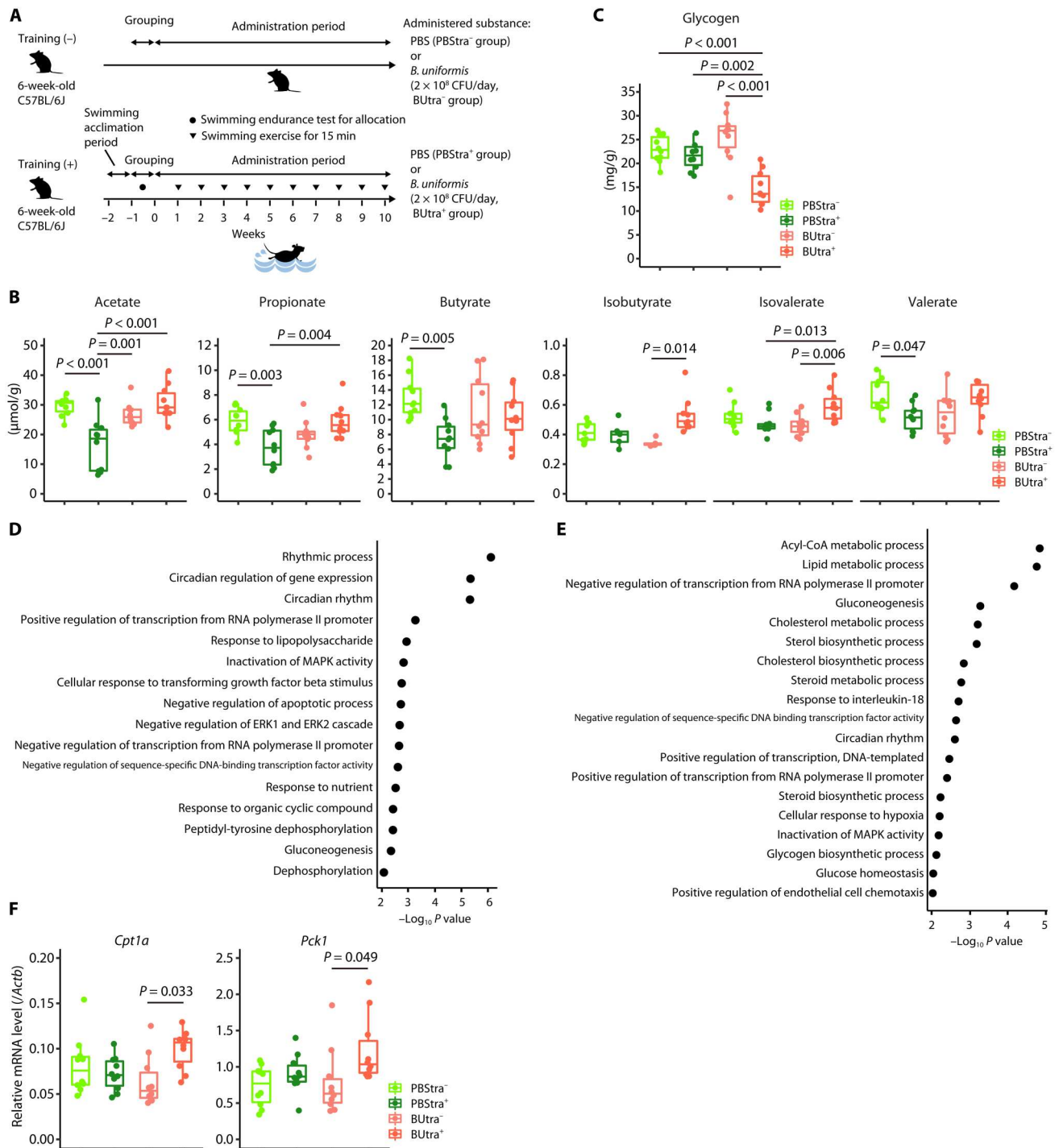
Because the cecal SCFA concentrations and hepatic glycogen levels of the BUtra<sup>+</sup> mice differed from those of the PBStr<sup>+</sup> mice (Fig. 5, B and C), we used *B. uniformis*-monoassociated mice to examine whether *B. uniformis* induces SCFA production in the murine intestines and the reduction of glycogen in the murine liver during exercise. Germ-free grade male C57BL/6N mice (10 weeks old) were injected with a single dose of *B. uniformis* type strain ( $2 \times 10^9$  CFU) to generate *B. uniformis*-monoassociated mice; three weeks after dose administration, fecal samples were collected, and the mice and germ-free mice were released from the sterile

isolator and allowed to swim in the flowing water pool for 20 min or until exhaustion. Immediately after swimming, the mice were dissected, and livers were collected. We observed that *B. uniformis* promoted acetate, propionate, isobutyrate, and isovalerate production but not butyrate and valerate in the murine intestine (Fig. 6A). These findings and the results from the aforementioned experiments that *B. uniformis* administration prevented the exercise-induced reduction of SCFA concentrations in the cecum of SPF mice imply a possibility that the presence of *B. uniformis* in the gut may induce the production of acetate and propionate at a higher rate than their reduction during exercise. In addition, similar to BUtra<sup>+</sup> mice, hepatic glycogen levels in the exercised *B. uniformis*-monoassociated mice were significantly lower than in exercised germ-free mice ( $P = 0.022$ ; Fig. 6B). Liver *Cpt1a* transcription was significantly up-regulated in the exercised *B. uniformis*-monoassociated mice compared with exercised germ-free mice ( $P = 0.009$ ), whereas *Pck1* transcription in the livers was similar in both groups (Fig. 6B).

### DISCUSSION

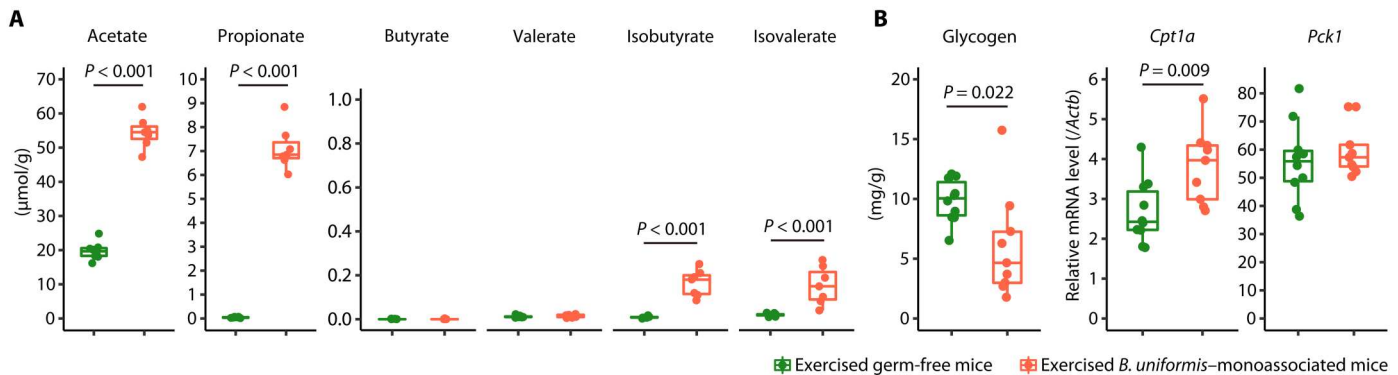
This study focused on *B. uniformis* because the fecal abundance of *B. uniformis* was correlated with the 3000-m race time in the athlete group. We showed that  $\alpha$ CD, a preferred substrate for *B. uniformis*, increased the abundance of *B. uniformis* and improved human and mouse endurance exercise performance. We tested the hypothesis that *B. uniformis* in the gut may improve endurance exercise performance by administering the bacteria to SPF mice and observing an improvement in their endurance exercise ability. To our knowledge, this is the first study to investigate the effect of the dominant gut microbe, *B. uniformis*, on endurance exercise performance.

In our animal exercise tests, SPF mice subjected to exercise after PBS administration presented reduced cecal SCFA levels, whereas SPF mice subjected to exercise following the administration of *B. uniformis* did not exhibit this reduction. Furthermore, *B. uniformis* administration markedly increased the acetate and propionate levels in the intestines of germ-free mice. These findings suggest that the presence of high levels of *B. uniformis* in the gut induces increased acetate and propionate production through carbohydrate metabolism at rates that exceed the rates of decrease induced by exercise, which results in the maintenance of SCFA concentrations. Notably, Scheiman *et al.* (9) showed that the intrarectal administration of propionate improves endurance and exercise performance of mice. In our experiments, although no metabolome changes were observed in the muscle of SPF mice subjected to exercise following *B. uniformis* administration, their livers showed elevated *Cpt1a* and *Pck1* mRNA levels, which encode rate-limiting enzymes in  $\beta$ -oxidation and gluconeogenesis, respectively, suggesting that  $\beta$ -oxidation and gluconeogenesis may be activated in the murine liver. The expression of these genes reportedly increases after the cecal infusion of propionate in the pig liver (29), and acetate administration also increases *Cpt1a* expression in the murine liver (30). Furthermore, fatty acid  $\beta$ -oxidation in the livers produces NADH (reduced form of nicotinamide adenine dinucleotide) and ATP, which are used for hepatic gluconeogenesis (31, 32). Another study showed that more than 60% of propionate directly infused in the cecum of mice is used in glucose production, which indicates its role as a gluconeogenic substrate (33). Focusing on hepatic glycogen, *B. uniformis* did not affect the glycogen levels in mice without exercise,



**Fig. 5. *B. uniformis* administration altered intestinal SCFA concentrations and hepatic glycogen levels in SPF mice.** (A) Experimental schedule. The mice were orally administered PBS or *B. uniformis* ( $2 \times 10^8$  CFU/day) for 10 weeks, and the mice in training (+) groups were subjected to weekly swimming exercises. (B) SCFA concentrations in the cecal content of PBS-administered mice subjected (PBSTra<sup>+</sup>,  $n = 10$ ) and not subjected to exercise (PBSTra<sup>-</sup>,  $n = 10$ ) and *B. uniformis*-administered mice subjected (BUtra<sup>+</sup>,  $n = 10$ ) and not subjected to exercise (BUtra<sup>-</sup>,  $n = 10$ ). (C) Hepatic glycogen content in PBSTra<sup>-</sup> ( $n = 10$ ), PBSTra<sup>+</sup> ( $n = 10$ ), BUtra<sup>-</sup> ( $n = 10$ ), and BUtra<sup>+</sup> ( $n = 10$ ) mice. (D and E) Gene ontology (GO) analysis results of differentially expressed genes in the liver. The enriched GO terms identified using DAVID are listed in (D) [PBSTra<sup>-</sup> ( $n = 6$ ) versus PBSTra<sup>+</sup> ( $n = 6$ )] and (E) [BUtra<sup>-</sup> ( $n = 6$ ) versus BUtra<sup>+</sup> ( $n = 6$ )]. Statistical significance was analyzed using Fisher's exact test.  $P < 0.01$  was considered significant. (F) Reverse transcription qPCR analysis of the hepatic *Cpt1a* and *Pck1* expression in PBSTra<sup>-</sup> ( $n = 10$ ), PBSTra<sup>+</sup> ( $n = 10$ ), BUtra<sup>-</sup> ( $n = 10$ ), and BUtra<sup>+</sup> ( $n = 10$ ) mice. The box-and-whisker plot illustrates the distribution of values within each group (B, C, and F). In (B), the Tukey-Kramer test was used for the statistical analysis of SCFAs, with the exception of isobutyrate, which was analyzed using the Steel-Dwass test. In (C), the Tukey-Kramer test was used for the analysis. In (F), the Tukey-Kramer test and Steel-Dwass tests were used for the analyses of *Cpt1a* and *Pck1*, respectively. These tests were selected on the basis of the outcomes of the normality test. All statistical tests were two-tailed.





**Fig. 6. Fecal SCFA and hepatic glycogen levels differed between exercised *B. uniformis*-monoassociated mice and germ-free mice.** (A) SCFA concentrations in the feces of exercised germ-free mice ( $n = 8$ ) and *B. uniformis*-monoassociated mice ( $n = 7$ ). (B) Hepatic glycogen content and expression of *Cpt1a* and *Pck1* in exercised germ-free mice ( $n = 8$ ) and *B. uniformis*-monoassociated mice ( $n = 7$ ). The box-and-whisker plot illustrates the distribution of values within each group. An unpaired *t* test was used to statistically analyze the acetate, propionate, isovalerate, glycogen, and *Cpt1a* levels. The Mann-Whitney *U* test was used to analyze the valerate, isobutyrate, and *Pck1* levels. These tests were selected on the basis of the outcomes of the normality test. All statistical tests were two-tailed.

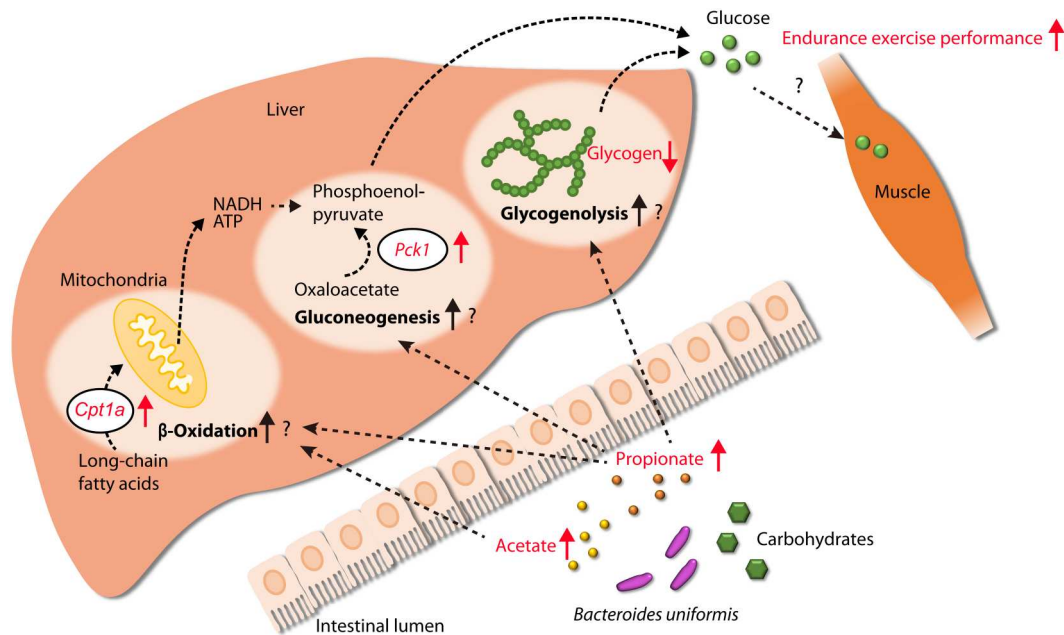
but its presence led to a reduction in glycogen in mice with exercise. Similarly, exercised *B. uniformis*-monoassociated mice had low hepatic glycogen. These results suggest that *B. uniformis* administration could not inhibit glycogen synthesis in the liver but induced hepatic glycogenolysis during exercise. In mice, intraperitoneally injected propionate stimulates hepatic glycogenolysis and increases blood glucose by activating the sympathetic nervous system (34). Therefore, the presence of *B. uniformis* in the gut, likely through stimulation by propionate, may enhance hepatic glycogenolysis during exercise. Together, these findings and previous reports suggest the potential existence of a mechanism through which exercise performance is improved by hepatic endogenous glucose production that is induced by the maintenance of the acetate and propionate concentrations in the gut as a result of bacteria (perhaps *B. uniformis*)-induced acetate and propionate production. We postulated a potential mechanism by which *B. uniformis* improves endurance exercise performance (Fig. 7), but further research is needed to determine the actual mechanism.

In a randomized, double-blind, placebo-controlled, parallel-group study, an 8-week  $\alpha$ CD supplementation improved the exercise bike time trials compared with baseline and placebo results but did not affect  $\dot{V}O_2\max$ . These phenotypes suggest that factors other than  $\dot{V}O_2\max$ , such as exercise economy, lactate threshold, and power, affect exercise performance (35). Consistent with our results, prior studies have shown that free radical scavengers (36), nitrates (37), and L-citrulline (38) supplementation can improve time trial performance without affecting  $\dot{V}O_2\max$ . Unidentified gut microbes other than *B. uniformis* affected by  $\alpha$ CD may also explain the improved cycling time trial performance in the  $\alpha$ CD group. However, energy from the test tablet is unlikely to affect the performance, as the energy of tablets used to supplement placebo, FL, or  $\alpha$ CD were approximately equal. The mechanisms behind the beneficial effects of  $\alpha$ CD supplementation in humans, such as weight loss in overweight people, are unclear (39–42). Therefore, identifying the mechanism through which  $\alpha$ CD improves endurance exercise performance in humans is challenging; however, we postulate that an increase in *B. uniformis* abundance may be partially responsible. Supplementation with FL did not improve human exercise bike time trials, unlike  $\alpha$ CD, but improved

mice swimming performance and increased *B. uniformis* in human feces. The lack of FL effects on endurance exercise performance may be because of differences in individual adaptability to the sport and physical conditions on the trial day. These confounding variables may have hampered the performance of the FL group, rendering the detection of their beneficial effects challenging. In addition, insufficient *B. uniformis* increase is also a potential reason. The increase of *B. uniformis* in the FL group may not be sufficient to improve exercise performance noticeably.

This study had a few limitations. First, the cross-sectional study observing long-distance runners had a few biases. The athletes included in the study had a higher *B. uniformis* abundance in the gut than nonathletes, but the two groups had different diets and living environments, which may affect gut microbiota composition (43, 44). Controlled diets and living environments are needed to determine whether a high abundance of *B. uniformis* is a characteristic of the gut microbiota of Japanese long-distance runners. In addition, our DNA extraction method using bead-beating, phenol-chloroform-isoamyl, and the glass fiber fleece-based purification columns may not completely remove PCR inhibitors such as polysaccharides (15) from the feces, which may explain PCR failure in five athletes and two nonathletes. Thus, future research focusing on the gut microbiome of Japanese athletes should use a DNA extraction method optimized for Japanese fecal samples, such as the method described by Tourlousse *et al.* (45). Second, our placebo-controlled study revealed that  $\alpha$ CD supplementation improves human exercise performance, but whether the effect was due to *B. uniformis* in the gut could not be confirmed. After confirming the safety of *B. uniformis* in humans, further exploration of the effects of *B. uniformis* on exercise in humans can be conducted using *B. uniformis* supplementation. Last, we conducted both the mice experiments and human studies involving only males; thus, the findings may not apply to females because of sex differences in athletic ability.

In conclusion, our findings suggest that *B. uniformis*, a dominant human gut bacterium, improves mice endurance exercise performance and that a substrate that *B. uniformis* prefers,  $\alpha$ CD, may be an effective prebiotic that enhances performance. However, the precise mechanisms through which *B. uniformis* enhances



**Fig. 7. Potential mechanism through which *B. uniformis* enhances host endurance exercise performance.** *B. uniformis* produces acetate and propionate in the intestine through carbohydrate metabolism. These SCFAs may then migrate to the liver and stimulate *Cpt1a* and *Pck1* gene expression, encoding enzymes involved in fatty acid oxidation and gluconeogenesis (29, 30), respectively. Specifically, propionate is used as a gluconeogenic substrate (33) and stimulates glycogenolysis (34), resulting in glucose production, which can be used as an energy source during exercise in exercise-related organs, such as muscle. Red arrows indicate the phenomena observed in this study.

endurance exercise performance are unclear. We hypothesize that acetate and propionate, associated with increased *B. uniformis* abundance in the gut, may facilitate glucose supply from the liver during exercise. Future studies are needed to clarify how *B. uniformis* improves endurance exercise performance, and elucidating the mechanism may pave the way for applications improving endurance exercise performance through gut microbiota modifications.

## MATERIALS AND METHODS

### Gut microbiome analysis of Japanese long-distance runners

#### Study design and participants

This cross-sectional study was conducted from July to September 2015. Forty-eight male Japanese long-distance runners (aged 18 to 22 years, defined as the athlete group) from the ekiden team of the Aoyama Gakuin University Track and Field Club participated in the study. The outcomes from the athletes were the fecal microbiota, blood parameters [hemoglobin concentration (Hb), mean corpuscular hemoglobin (MCH), MCH concentration (MCHC), mean corpuscular volume (MCV), hematocrit, red blood cell (RBC) count, creatine phosphokinase (CPK), hematocrit, serum iron (Fe), and ferritin], and their running time in a 3000-m race. No eligibility or exclusion criteria were set; thus, all the team members (i.e., 48 athletes) participated in the study. They resided in team dormitories in Tokyo or Kanagawa Prefecture. Ten Japanese nonathlete healthy males (aged 22 to 24 years, defined as the nonathlete group), who were not related or socially associated with the ekiden team members and lived in individual houses in Tokyo or Kanagawa, participated in this investigation for comparison purposes. The exclusion criteria for the nonathletes were as follows: (i) past or

present serious heart, liver, kidney, or gastrointestinal disease; (ii) allergy to any medications or foods; (iii) current disease requiring treatment; (iv) consumption of dietary supplements; (v) current participation in other clinical tests, participation within the previous 4 weeks, or plans to participate; and (vi) subjects whom a physician determined to be unable to follow instructions because of personality or health concerns. All participants gave informed consent to participate in the investigation before enrollment. In addition, if the participant was a minor (age < 20 years in Japan), then his parents provided consent.

#### Ethics statements

The protocol for investigating athletes was approved by the Ethical Committee for Research in Humans of Shiba Palace Clinic (Tokyo, Japan) in compliance with the Helsinki Declaration (approval no. 2015 AC-001). The protocol for investigating the nonathlete gut microbiota was approved by the Ethical Committee for Research in Humans of Aisei Hospital Ueno Clinic (Tokyo, Japan) in compliance with the Helsinki Declaration (approval no. 27030).

#### Fecal microbiota analysis

Fecal samples of both athletes and nonathletes were collected using the same collection protocol in July 2015 and September 2015, respectively. Participants collected fecal samples at their convenience in fecal collection containers (Sarstedt, Nümbrecht, Germany) at their dormitories or homes. After collection, the samples were frozen and delivered to our laboratory while still frozen and maintained at  $-30^{\circ}\text{C}$  for later use. Subsequently, 0.2 g of each thawed fecal sample was aliquoted into plastic tubes. The samples were washed in 1.0 ml of PBS buffer and centrifuged at 14,000g for 3 min. The pellets were suspended in 500  $\mu\text{l}$  of extraction buffer [166 mM tris-HCl, 66 mM EDTA, and 8.3% (w/v) SDS (pH 9.0)]

and tris-EDTA (TE) buffer-saturated phenol. Glass beads (300 mg; diameter, 0.1 mm) were added to the suspension, and the mixture was vigorously vortexed for 60 s using a Multi-beads Shocker (Yasui Kikai, Osaka, Japan). After centrifugation at 14,000g for 5 min, 400  $\mu$ l of the supernatant was purified using phenol-chloroform-isoamyl alcohol, and DNA was precipitated with isopropanol. The samples were washed in 70% ethanol and dissolved in TE buffer. The High Pure PCR Template Preparation Kit (Roche Diagnostics, Rotkreuz, Switzerland) was used for purification according to the manufacturer's recommendations. Sequencing of the V4 region of the 16S rRNA-encoding gene was performed according to the manufacturer's instructions. Briefly, 12.5 ng of purified DNA from each sample was used to amplify the V4 region of the 16S rRNA-encoding genes using PCR (23 cycles) with 515F and 806R primers (46). Amplicons were purified using the AMPure XP system (Beckman Coulter Biomedical GmbH, Munich, Germany), and double barcodes were then added on both the forward and reverse strands via a second round of PCR (eight cycles). The double-barcoded amplicons were purified using the AMPure XP system, pooled with a 1 pM PhiX library, and sequenced using a MiSeq system (Illumina, San Diego, CA, USA). Because of insufficient V4 region amplification, five participants in the athlete group and two in the nonathlete group were excluded from the gut microbiota analysis. QIIME version 1.9.1 was used for sequence filtering and analysis (47). Sequences with a quality score of less than 29 were removed from the fastq files. Chimeric sequences were filtered out using USEARCH, and operational taxonomic units (OTUs) were assigned using an open-reference OTU picking process with a 97% pairwise identity threshold. The  $\alpha$ -diversity (Chao1 richness estimates, Shannon diversity index, and PD of the whole tree) within two groups and the distances between participants (unweighted and weighted UniFrac distances as a measurement of the  $\beta$ -diversity) were also estimated using QIIME with data rarefied at 35,000 reads per sample. The  $\beta$ -diversity was visualized using principal coordinates analysis. The LEfSe analysis was performed using the online tool LEfSe (Galaxy version 1.0), available at the following website: <http://huttenhower.sph.harvard.edu/galaxy/> (48). The species-specific primers and TaqMan probes shown in table S2, reported previously (49, 50) or designed for this study, were used for qPCR. A LightCycler 480 Instrument II (Roche Diagnostics) was used to amplify and detect specific sequences. The standard curve was generated by amplifying 10-fold serial dilutions of targeted bacterial 16S rRNA-encoding gene sequences synthesized by Thermo Fisher Scientific (Waltham, MA, USA).

### Blood analyses

During the same period as that at which fecal sampling was performed, blood samples from athletes were collected by a physician in a clinic. Before blood sample collection, no dietary or behavioral restrictions were given to the participants. Blood samples were collected in ethylenediaminetetraacetic acid disodium salt dihydrate-coated tubes for Hb, MCH, MCHC, MCV, hematocrit, and RBC analyses and in noncoated tubes for CPK, Fe, and ferritin analyses. The collected blood samples were sent directly to a clinical laboratory.

### The 3000-m race

The time required by each participant to run 3000 m on the outdoor field track of Aoyama Gakuin University was recorded. Twenty-five of 48 athletes participated in this trial, but the other 23 athletes

refused the measurement of their 3000-m race time. All participating athletes started at the same time.

### Statistical analysis

To compare the gut microbial  $\alpha$ - and  $\beta$ -diversities between groups, we used a two-sample *t* test with Monte Carlo permutations and permutational multivariate analysis of variance (PERMANOVA), respectively. The number of permutations for both tests was set to 999 to calculate *P* values. Correlations among the relative abundances of *Bacteroides*, *Prevotella*, *Escherichia*, *Lachnospira*, and *Sutterella* and blood data or the 3000-m race time of the athletes were determined using the Pearson correlation coefficient. Correlations among the log<sub>10</sub> copy numbers of each *Bacteroides* and *Phocaeicola* species and the 3000-m race time were determined using the Pearson correlation coefficient. In these correlation analyses, missing data were deleted by pairwise deletion. Mann-Whitney *U* tests were used to compare the relative abundances of genera and log<sub>10</sub> copy numbers of each *Bacteroides* and *Phocaeicola* species between the athlete and nonathlete groups. These statistical analyses were conducted using SPSS Statistics for Windows (version 23; IBM, Armonk, NY, USA) and R (version 4.0.2; R Foundation for Statistical Computing, Vienna, Austria). Differences with *P* < 0.05 were considered statistically significant. In the box plots, the center line represents the median, and the box boundaries represent the 25th and 75th percentiles. The whiskers correspond to the most extreme values within 1.5 times the interquartile range below the 25th percentile and above the 75th percentile.

### Randomized, double-blind, placebo-controlled, parallel-group study with FL or $\alpha$ CD as test supplements Study design

This study was a randomized, double-blinded, placebo-controlled, parallel-group study conducted at the Chiyoda Paramedical Care Clinic, Tokyo, Japan. Safety and adverse events were monitored by hematology, vital signs, and individual self-reporting at each clinic visit.

### Ethics statement

This study was conducted in accordance with the Declaration of Helsinki and Ethical Guidelines for Medical and Health Research Involving Human Subjects (notification from the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labour, and Welfare). Before implementation, the Institutional Review Board of Chiyoda Paramedical Care Clinic reviewed and approved the study protocol (approval no.: 18071903; approval date: 20 July 2018). The study was registered in the University Hospital Medical Information Network system (registration ID: UMIN000033748; registration date: 14 August 2018). All the participants provided informed consent before a preliminary assessment ( $\dot{V}O_2$ max measurement; visit -4 weeks) and were enrolled between 17 and 19 August 2018. After the preliminary test, 36 participants were allocated to one of the three groups on 13 September 2018, and the supplementation period started on 20 to 25 September 2018 and ended on 22 to 27 November 2018.

### Participants and sample size

This study was a first clinical trial; therefore, no sample size calculation could be performed. Participants were recruited at the Chiyoda Paramedical Care Clinic. A total of 65 participants who consented to participate after receiving a thorough explanation of the nature of the experiment were enrolled in the study and underwent the preliminary assessment ( $\dot{V}O_2$ max measurement)



approximately 4 weeks before test supplement intake. After the  $\dot{V}O_2\text{max}$  measurement, four participants declined to participate in the study for personal reasons, and 25 met at least one of the exclusion criteria described below. Therefore, 36 Japanese males received a placebo, FL, or  $\alpha\text{CD}$  as a supplement for 9 weeks. During the study, the participants were asked to maintain their normal dietary and physical activity. One, two, and two participants in the placebo, FL, and  $\alpha\text{CD}$  groups, respectively, dropped out of the study for personal reasons. The placebo, FL, and  $\alpha\text{CD}$  groups had 11, 10, and 10 participants in the final analysis, respectively. The inclusion criteria were as follows: (i) healthy males aged 20 to 49 years at the time of providing consent; (ii) a regular exercise habit, that is, one to two sessions ( $\geq 30$  min per session) per week of exercise of  $\geq 5$  metabolic equivalents (METs), excluding resistance (weight) training among the exercises listed in the 2011 Compendium of Physical Activities (51); (iii) continuation of exercise habits during the study period; and (iv) verbal agreement not to consume prohibited foods (listed below) for at least 1 week before the date of providing consent and throughout the study period. The exclusion criteria were as follows: (i) self-report of  $\leq 5$  bowel movements per week or proneness to diarrhea; (ii) past or present serious heart, liver, kidney, or gastrointestinal disease; (iii) high alcohol consumption (60 g/day); (iv) extremely irregular eating habits or irregular daily rhythm due to shifting or night work; (v) allergy to any medicines or foods; (vi) current participation in other clinical tests, participation within the past 4 weeks, or plans to participate; (vii) blood donation of more than 200 ml in the past month or over 400 ml in the past 3 months; and (viii) subjects who were determined by a physician to be unable to follow instructions or complete exercise sessions because of their personality or health concerns.

#### Randomization and blinding

Randomization and allocation were carried out by Okutoeru LLC (Tokyo, Japan).  $\dot{V}O_2\text{max}$  and age were used to stratify 36 participants into three groups (12 participants per group) through stratified randomization (Fig. 2A). The Microsoft Excel randomization function generated the allocation sequence. The study investigators, research coordinators, physicians, clinical staff, and participants were blinded to the allocation of the participants to the treatment groups. The test supplements were provided in an opaque sachet with a numeric code, and each participant's samples were blinded using a specific code that did not link to the potential treatment.

#### Supplements

The tested substances were administered to the participants as tablets prepared by Sankyo Co. (Tokyo, Japan). Tablets containing NIPPN flaxseed lignans (Nippon Flour Mills, Tokyo, Japan), a source of dietary SDG, were designated the FL supplements. NIPPN flaxseed lignans contained approximately 40% (w/w) SDG and 40% (w/w)  $\alpha\text{CD}$ . Tablets containing Dexyppearl- $\alpha$  (Ensuiko Sugar Refining, Tokyo, Japan), an almost pure  $\alpha\text{CD}$  ( $>98\%$ ), were designated the  $\alpha\text{CD}$  supplements. The placebo tablet contained maltitol instead of NIPPN flaxseed lignans or Dexyppearl- $\alpha$ . Table S3 details the compositions of the placebo, FL supplement, and  $\alpha\text{CD}$  supplement.

#### Study interventions

All participants who were allocated to one of the three groups ingested the corresponding test supplements (three tablets) daily for 9 weeks beginning on the first day of the experiment. The three tablets were ingested with water at the same time. Participants in the three groups were fed a placebo (placebo group), FL (200 mg/

day, FL group), or  $\alpha\text{CD}$  (200 mg/day,  $\alpha\text{CD}$  group) as the supplement. Participants received a prescribed dinner on the days before clinic visits (300 g of white rice, a cup of miso soup, a Hamburg steak, and gomoku-ni). On clinic visit days, they received a prescribed breakfast ("cream and brown rice bran," a nutritional supplement). These meals were delivered to the participants a few days before their consumption.

#### Prohibited foods

The following foods were prohibited: (i) foods and beverages that improve exercise performance; (ii) pharmaceuticals and quasi-drugs that are used to promote recovery or prevent and improve "fatigue" or "physical strength"; and (iii) dietary supplements (including "Foods with Functional Claims" and "Food for Specified Health Uses"). All participants were prohibited from consuming these foods starting at least 1 week before the start of the supplementation period until the end of the study.

#### Study outcomes

The primary outcomes were the  $\dot{V}O_2\text{max}$  and questionnaire responses regarding fatigue. The secondary outcomes were physical performance, VT, RPE, blood biochemistry, muscle and body fat mass, and gut microbiota composition. The participants allocated to one of the three groups were subjected to the designated tests (described below) at baseline (0) and after 4, 8, and 9 weeks at the Chiyoda Paramedical Care Clinic. The clinic staff collected the data. A physician monitored the condition of the participants during all tests. The assessments and visit schedules at  $-4$ , 0, 4, 8, and 9 weeks are depicted in Fig. 2B. Every participant who attended a visit completed all exercise tests.

#### $\dot{V}O_2\text{max}$ , VT, and maximum exercise intensity (performed on the visits at $-4$ and 9 weeks)

Exercise testing was conducted on an exercise bike (AEROBIKE-75XLIII; Konami Sports Life, Tokyo, Japan) at room temperature ( $23^\circ \pm 2^\circ\text{C}$ ) with  $45 \pm 5\%$  relative humidity. The exercise load was incrementally increased by 15 W every minute until exhaustion. The oxygen and carbon dioxide concentrations in expired gas during exercise were measured using an Aero Monitor expired gas analyzer (AE-310s; Minato Medical Science Co., Osaka, Japan). The heart rate was monitored during the exercise. Exhaustion was determined on the basis of the following criteria: (i) The participant declared himself exhausted or was unable to continue exercising; (ii) the participant's heart rate reached the estimated maximum for his age, defined as  $220 - \text{age}$ ; or (iii) a physician declared the participant exhausted. The maximum oxygen consumption and pedal resistance immediately before exhaustion were defined as the  $\dot{V}O_2\text{max}$  and maximum exercise intensity, respectively.

#### Fatigue questionnaire and RPE (performed on the visits at 0, 4, and 8 weeks)

The exercise testing was conducted with an exercise bike as described above in a room maintained at  $23^\circ \pm 2^\circ\text{C}$  with  $45 \pm 5\%$  relative humidity. The pedaling resistance of the exercise bike was set to 45% of each participant's maximum exercise intensity during testing. The participants maintained a pedal speed as close as possible to 60 rpm for 50 min. The RPE was measured with Borg's RPE scale at 0, 10, 20, 30, 40, 45, and 50 min during the exercise. In addition, a VAS questionnaire was used to assess fatigue after exercise (0, 30, and 60 min after exercise). The participants completed the questionnaire themselves, and the questionnaire used in this

study was created by the Japanese Society of Fatigue Science ([www.hirougakkai.com/VAS.pdf](http://www.hirougakkai.com/VAS.pdf)).

#### **Exercise performance (performed on the visits at 0, 4, and 8 weeks)**

The effects of FL and  $\alpha$ CD on exercise performance were evaluated on the basis of the time needed to pedal 10 km on an exercise bike (FBS-101; Fujimori Ltd., Takaoka, Japan). This test was conducted at room temperature ( $23^{\circ} \pm 2^{\circ}\text{C}$ ) with  $45 \pm 5\%$  relative humidity. The intensity level (i.e., the resistance of the pedal) was set to 45% of each participant's maximum exercise intensity. The participants were asked to pedal as fast as possible for 10 km. The clinic staff measured the time.

#### **Blood testing (performed on the visits at 0, 4, and 8 weeks)**

Blood was collected immediately before and after exercise to measure lactate, CPK, lactate dehydrogenase, glucose, creatine, free fatty acids, cortisol, amino acids, glucagon, interleukin-6 (IL-6), growth hormone, and diacron-reactive oxygen metabolite (dROM) levels. Blood was collected 60 min after exercise to measure lactate dehydrogenase, CPK, creatine, glucose, and lactate levels. Blood samples were collected in ethylenediaminetetraacetic acid disodium salt dihydrate-coated tubes for amino acid analysis; aprotinin-coated tubes for glucagon analysis; heparin-coated tubes for glucose analysis; perchloric acid-added tubes for lactate analysis; and noncoated tubes for CPK, creatine, lactate dehydrogenase, free fatty acids, cortisol, IL-6, growth hormone, and dROM analysis. All analyses except the dROM analysis were carried out at LSI Medience Co. (Tokyo, Japan), while the dROM analysis was performed with FREE Carrio DUO (Wismerll Co., Tokyo, Japan) at our laboratory.

#### **Muscle and body fat mass (performed on the visits at 0, 4, and 8 weeks)**

These parameters were measured using a body composition analyzer (InBody 570; InBody Japan Inc., Japan) before a 50-min constant-load bike exercise.

#### **Gut microbiota (performed on the visits at 0, 4, and 8 weeks)**

Fecal samples were collected at 0 and after 4 and 8 weeks of supplementation. Each participant collected the samples in fecal collection containers (Sarstedt) at their houses within 5 days before each visit. After collection, the samples were frozen at the participants' houses, kept at approximately  $-30^{\circ}\text{C}$ , and delivered to the clinic by each participant. As in the cross-sectional study, DNA was extracted from the samples, and gut microbiota was analyzed using the MiSeq platform (Illumina). The total number of analyzed sequence reads was 4,757,361. The mean, minimum, and maximum reads per sample were 52,859, 12,805, and 125,205, respectively; thus, data rarefied at 10,000 reads per sample were used to estimate the  $\alpha$ - and  $\beta$ -diversities. *B. uniformis* abundance was measured using species-specific qPCR, as described above.

#### **Statistical analyses**

In this randomized, double-blind, placebo-controlled, parallel-group study, Mann-Whitney *U* tests were used to compare *B. uniformis* abundance between the two treatment groups and the placebo group, and Wilcoxon signed-rank tests were used to compare baseline and 4- or 8-week levels within each group. Except for microbial data, normally distributed continuous raw data were assessed using the Shapiro-Wilk test. All subsequent statistical tests were carried out on the basis of the results of normality tests. To determine the significance of the differences in parameters between the two treatment groups and the placebo group, an

unpaired *t* test or the Mann-Whitney *U* test was used. A paired *t* test or Wilcoxon signed-rank test was used to compare baseline and 4- or 8-week levels within each group. These statistical analyses were conducted using R (version 4.0.2; R Foundation for Statistical Computing). Differences of  $P < 0.05$  were considered statistically significant. In the box plots, the center line represents the median, and the box boundaries represent the 25th and 75th percentiles. The whiskers correspond to the most extreme values within 1.5 times the interquartile range below the 25th percentile and above the 75th percentile. Pie charts in fig. S3 show the mean relative abundance of each bacterial genus to illustrate gut microbial compositions. The participants' baseline data are shown in table S1, expressed as the mean and SD values.

#### **Mouse treatment, exercise regimen, and sample collection** ***B. uniformis* and *P. dorei* cultivation**

The *B. uniformis* JCM 5828 or *P. dorei* JCM 13471 type strains, provided by RIKEN BioResource Research Center (Ibaraki, Japan), were cultured on glucose blood liver (BL) agar plates (Nissui Pharmaceutical, Tokyo, Japan) or modified EG (Eggerth-Gagnon) agar plates (52) supplemented with 5% (v/v) horse blood for 48 hours at  $37^{\circ}\text{C}$  under anaerobic conditions [10% (v/v)  $\text{CO}_2$ , 4% (v/v)  $\text{H}_2$ , and 86% (v/v)  $\text{N}_2$ ], respectively. Colonies from the agar plates were collected and incubated overnight in 25 ml of Gifu anaerobic medium broth (Nissui Pharmaceutical) or modified EG medium broth supplemented with Fildes' peptic digest of horse blood [5% (v/v)] at  $37^{\circ}\text{C}$  under anaerobic conditions, respectively. The culture was diluted and plated on BL or modified EG with horse blood agar to count viable bacterial cells. After 48 hours of culture, colonies on the plates were manually counted, and the CFU per milliliter was calculated.

#### **Mouse housing**

SPF male C57BL/6J mice (Japan SLC, Shizuoka, Japan) were individually housed in open-top cages covered by wire bar lids and maintained on a 12-hour light/12-hour dark cycle at room temperature ( $22^{\circ} \pm 3^{\circ}\text{C}$ ) with feed and water provided ad libitum.

#### **Endurance exercise test**

Physical performance was evaluated using a flowing water pool (length, 92 cm; width, 45 cm; height, 45.5 cm; water depth, 35 cm; Antitec, Otsu, Japan) (53, 54) developed at Kyoto University. The pool had six lanes (length, 22.5 cm; width, 7.0 cm), and the water temperature was maintained at  $34^{\circ} \pm 0.5^{\circ}\text{C}$ . The mice were fed the chow diet CE-2 (CLEA Rodent Diet CE-2; CLEA Japan, Tokyo, Japan). After a 1-week acclimation period, SPF male C57BL/6J mice (aged 7 weeks) were used in the experiment. The mice were then given a 1-week swimming acclimation period. The acclimation swimming sessions were performed every 2 days and consisted of the following: On day 1, the flow rate was maintained at 8 liter/min for 3 min, increased by 1 liter/min every 3 min to 12 liter/min, and maintained at 12 liter/min for 3 min; on day 2, the flow rate was maintained at 10 liter/min for 5 min, increased by 1 liter/min every 5 min to 12 liter/min, and maintained at 12 liter/min for 5 min, and on day 3, the flow rate was maintained at 12 liter/min for 5 min, 13 liter/min for 15 min, and 14 liter/min for 15 min. During these swimming periods, the swimming was stopped if a mouse reached exhaustion (i.e., failure to return to the water surface within 3 s). Three days after swimming acclimatization, the swimming endurance of the mice was tested to divide them into appropriate groups. The swimming test consisted of a

flow rate program of 12 liter/min for 5 min, 13 liter/min for 5 min, and 14 liter/min until exhaustion. The time taken by each mouse to reach exhaustion, i.e., failure to return to the water surface within 3 s, was recorded. The mice were tested for their endurance levels and split into two groups with similar swimming times (PBS and *B. uniformis* or *P. dorei* treatment groups). The treatment group was orally administered *B. uniformis* JCM 5828<sup>T</sup> or *P. dorei* JCM 13471<sup>T</sup> ( $2 \times 10^8$  CFU, 200  $\mu$ l of PBS per mouse) daily for four consecutive weeks, whereas the control group received sterile PBS. During this administration period, mice were subjected to a weekly swimming endurance test under the same conditions as the initial swimming endurance test. These experiments were approved by the Institutional Animal Care and Use Committee of New Drug Research Center Inc. (approval no.: 170428A and 180130A; approval date: May 2017 and January 2018; experimental period: May to July 2017 and February to May 2018, respectively).

#### ***B. uniformis*– or PBS-administered mice with and without exercise**

After the acclimation period, SPF male C57BL/6J mice (aged 5 weeks) were introduced to the CE-2 chow diet and used in the experiment. The mice were allocated into exercising and non-exercising groups based on their body weight. The non-exercising group was further divided into two subgroups (PBStr<sup>-</sup> and BUtra<sup>-</sup>). After swimming acclimation, the exercise group mice were divided into two subgroups (PBStr<sup>+</sup> and BUtra<sup>+</sup>) with similar average swimming times. After grouping, mice in the *B. uniformis* treatment groups (BUtra<sup>-</sup> and BUtra<sup>+</sup>) were orally administered *B. uniformis* JCM 5828<sup>T</sup> ( $2 \times 10^8$  CFUs, 200  $\mu$ l of PBS per mouse) daily for 10 weeks, whereas those in the PBS groups (PBStr<sup>-</sup> and PBStr<sup>+</sup>) received sterile PBS (Fig. 5A). During this administration period, the PBStr<sup>+</sup> and BUtra<sup>+</sup> group mice had weekly swimming exercise sessions in the flowing water pool for 10 weeks (Fig. 5A). The weekly exercise consisted of a flow rate program of 12 liter/min for 5 min and 13 liter/min for 10 min. Immediately after the swimming exercise in week 10, mice were euthanized under isoflurane anesthesia, and their liver, cecal content, and quadriceps femoris were collected and frozen in liquid nitrogen. This experiment was approved by the Institutional Animal Care and Use Committee of Sankyo Labo Service Corporation Inc., Tokyo, Japan (approval no.: SKL-A-17095; approval date: February 2018; experimental period: February to May 2018).

#### **Administration of FL**

After a 1-week acclimation period, male C57BL/6J SPF mice (aged 7 weeks) were housed in the laboratory. Subsequently, the mice were subjected to a swimming acclimation period, a swimming endurance capacity test for allocation, and grouping using the endurance exercise test protocols. After grouping, mice were fed the chow control diet CE-2 alone or supplemented with 5% (w/w) FL for 7 weeks. NIPPON flaxseed lignans (Nippon Flour Mills) was used as the FL. During the administration period, mice were subjected to a weekly swimming endurance test. Fecal samples were collected at day 0 and week 7 and immediately stored at  $-80^{\circ}\text{C}$ . This experiment was performed following approval by the Institutional Animal Care and Use Committee of New Drug Research Center Inc. (approval no.: 171010D; approval date: October 2017; experimental period: October to December 2017).

#### **Administration of $\alpha$ CD**

The acclimation of the mice, exercise protocols, and fecal sampling were performed similarly to the administration of FL. For 7 weeks,

mice were fed the chow control diet CE-2 alone or supplemented with 5% (w/w)  $\alpha$ CD. Dexypersulfate- $\alpha$  (Ensui Sugar Refining) was used in the study. This experiment was performed following approval by the Institutional Animal Care and Use Committee of Asahi Quality and Innovations Ltd. (approval no.: 18-21-01; approval date: May 2018; experimental period: May to August 2018).

#### ***B. uniformis*–monoassociated mice**

Germ-free male C57BL/6N mice (aged 10 weeks; three to four animals per cage; Sankyo Labo Service Co.) were housed and maintained in a vinyl isolator at a constant temperature ( $23^{\circ} \pm 2^{\circ}\text{C}$ ). After 6 days of acclimation, on day 0, mice in the *B. uniformis*–monoassociated group were given a single oral dose of *B. uniformis* JCM 5828<sup>T</sup> ( $2 \times 10^9$  CFUs, 200  $\mu$ l of PBS per mouse), while those in the control group (germ-free mouse group) received sterile PBS. On days 0 and 21 (the final day of the experiment), fecal samples were collected and immediately stored at  $-80^{\circ}\text{C}$ . On day 21, the *B. uniformis*–monoassociated and germ-free mice were removed from the isolator and allowed to swim for 20 min or until exhaustion in the flowing water pool with six lanes (water temperature:  $34^{\circ} \pm 0.5^{\circ}\text{C}$ ). The flow rate was maintained at 10 liter/min for 5 min, 11 liter/min for 5 min, and 12 liter/min for 10 min. During these exercises, swimming was stopped if a mouse reached exhaustion (i.e., failure to return to the water surface within 3 s). Immediately after the swimming, the mice were euthanized under isoflurane anesthesia, and their livers were collected, frozen, and stored at  $-80^{\circ}\text{C}$ . The experiment was conducted following approval from the Institutional Animal Care and Use Committee of Sankyo Labo Service Corporation Inc. (approval no.: SKL-J18004; approval date: April 2018; experimental period: April to May 2018).

#### **Fecal microbiota analysis**

Fecal DNA was extracted from 50 mg of mice fecal samples using the same protocol described above for the human fecal samples. The quantification of *B. uniformis* using qPCR and 16S rRNA-encoding gene sequencing with MiSeq platform (Illumina) was also performed using the same protocol for human fecal samples.

#### **RNA sequencing**

Six mice from each group (PBStr<sup>-</sup>, PBStr<sup>+</sup>, BUtra<sup>-</sup>, and BUtra<sup>+</sup>) were randomly selected. Total liver RNA was extracted using the RNeasy Mini Kit (QIAGEN, Venlo, the Netherlands), and the subsequent steps were performed at Bioengineering Lab. Co. Ltd. (Sagamihara, Japan). Briefly, cDNA libraries were constructed using the KAPA Stranded RNA-Seq Kit (Kapa Biosystems, Wilmington, MA, USA) and FastGene Adapter Kit (Nippon Genetics, Tokyo, Japan), following the manufacturer's protocol. The fragments were sequenced on the HiSeq X System (Illumina). For data analysis, read sequences (75 base pairs) were aligned to the *Mus musculus* (GRCm38.p6) reference genome ([www.ncbi.nlm.nih.gov/assembly/GCF\\_000001635.26/](http://www.ncbi.nlm.nih.gov/assembly/GCF_000001635.26/)) using HISAT2 version 2.1.0 (55). The gene-mapped reads in the reference sequence were counted using featureCounts version 1.6.3 (56). Differentially expressed genes were detected using edgeR version 3.20.9 following iDEGES normalization (57, 58). GO analysis was conducted using the DAVID website (<https://david.ncicrf.gov/>) (59).

#### **Reverse transcription qPCR**

Total RNA from the liver or quadriceps femoris was extracted using the RNeasy Mini Kit (QIAGEN) following the manufacturer's instructions. cDNA was synthesized using the PrimeScript RT Reagent Kit (Takara Bio, Kusatsu, Japan). Reverse transcription qPCR was performed using the LightCycler 480 System (Roche



Diagnostics) and TB Green Premix Ex Taq II (Takara Bio). The primer sequences for *Pck1*, *Cpt1a*, *Hk1*, *Hk2*, *Glut4*, and *Pgc-1a* have been reported previously (60–64). For *Pygm*, the Pygm Mouse qPCR Primer Pair (OriGene Technologies, Rockville, MD, USA) was used. The relative amounts of these mRNAs were normalized using *Actb* mRNA.

### SCFA analysis

The SCFA measurements were performed at TechnoSuruga Laboratory Co. Ltd. (Shizuoka, Japan). We analyzed 100 mg of the cecal content or fecal samples using a gas chromatography–flame ionization detector system (7890B Gas Chromatograph System, Agilent Technologies, Santa Clara, CA, USA) fitted with a DB-WAXetr column (30 m, 0.25-mm internal diameter, 0.25- $\mu$ m film thickness; Agilent Technologies) to measure SCFA levels, as previously reported (65).

### Glycogen content

The liver and muscle glycogen content was measured using a Glycogen Colorimetric/Fluorometric Assay Kit (BioVision, Abcam, Waltham, MA, USA) according to the manufacturer's instructions. Briefly, 10 mg of liver or quadriceps femoris was homogenized in 200  $\mu$ l of cooled water and boiled for 10 min. The boiled samples were then centrifuged, and glucoamylase was added to the diluted supernatants to hydrolyze the glycogen into glucose. During a 30-min incubation at room temperature, glucose was oxidized with an OxiRed probe (Abcam) to generate color, and absorbance (optical density at 570 nm) was measured using a Synergy HTX Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA).

### Metabolite extraction and CE-TOFMS measurements

A quantitative analysis of charged metabolites in the quadriceps femoris was performed using CE-TOFMS as described previously (66, 67). Quadriceps femoris muscle samples (50 mg) were submerged in methanol (500  $\mu$ l) containing internal standards [methionine sulfone (Wako, Tokyo, Japan), 2-(*N*-morpholino)ethane sulfonic acid (Dojindo Laboratories, Tokyo, Japan), and *D*-camphor-10-sulfonic acid (Wako) each at 20  $\mu$ M]. The mixture was combined with four 3-mm zirconia beads (BioSpec Products, Bartlesville, OK, USA) and vigorously shaken for 5 min using a Shake Master NEO (Biomedical Science, Tokyo, Japan). Subsequently, deionized water (200  $\mu$ l) and chloroform (500  $\mu$ l) were added, and the suspension was vigorously shaken for 5 min and centrifuged at 4600g and 4°C for 30 min. The supernatant was filtered through a 5-kDa-cutoff filter column (Millipore, Burlington, MA, USA). The filtrate was concentrated via centrifugation (3 hours at 40°C) and dissolved in water containing reference compounds [200  $\mu$ M each of 3-aminopyrrolidine (Sigma-Aldrich, St. Louis, MO, USA) and trimesic acid (Wako)] before CE-TOFMS analysis. As described previously (68), the CE-TOFMS experiments were performed using an Agilent CE-TOFMS system (Agilent Technologies).

### Statistical analyses

Mann-Whitney *U* tests were used to compare *B. uniformis* or the relative abundance of *Bacteroides* across the groups. The Benjamin-Hochberg procedure was used to estimate RNA-seq false discovery rates (*q* values). The raw data from the other mouse experiments were tested for normality using the Shapiro-Wilk test, and subsequent tests were selected on the basis of the outcomes. The swimming endurance across the groups was assessed using the Mann-Whitney *U* test. The cecal SCFA (excluding isobutyrate); hepatic glycogen; liver *Cpt1a* transcript; and muscle *HK2*, *Pygm*,

and *Pgc1a* levels were assessed using the Tukey-Kramer test. The isobutyrate, liver *Pck1* transcript, muscle glycogen, and muscle *Hk1* and *Glut4* levels were assessed using the Steel-Dwass test. Acetate, propionate, and isovalerate in feces and glycogen and *Cpt1a* in the livers were compared between germ-free and *B. uniformis*-monoassociated mice using an unpaired *t* test; valerate, isobutyrate, and *Pck1* levels were compared using the Mann-Whitney *U* test. These statistical analyses were conducted using SPSS (version 23; IBM) and R (version 4.0.2; R Foundation for Statistical Computing). Differences of *P* < 0.01 or 0.05 were considered statistically significant in GO analyses or other experiments. In the box plots, the center line represents the median, and the box boundaries represent the 25th and 75th percentiles. The whiskers correspond to the most extreme values within 1.5 times the interquartile range below the 25th percentile and above the 75th percentile.

## Supplementary Materials

### This PDF file includes:

Figs. S1 to S10

Tables S1 to S3

[View/request a protocol for this paper from Bio-protocol.](#)

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