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Supplementary Materials for

Bacteroides uniformis and its preferred substrate, α-cyclodextrin, enhance endurance exercise performance in mice and human males

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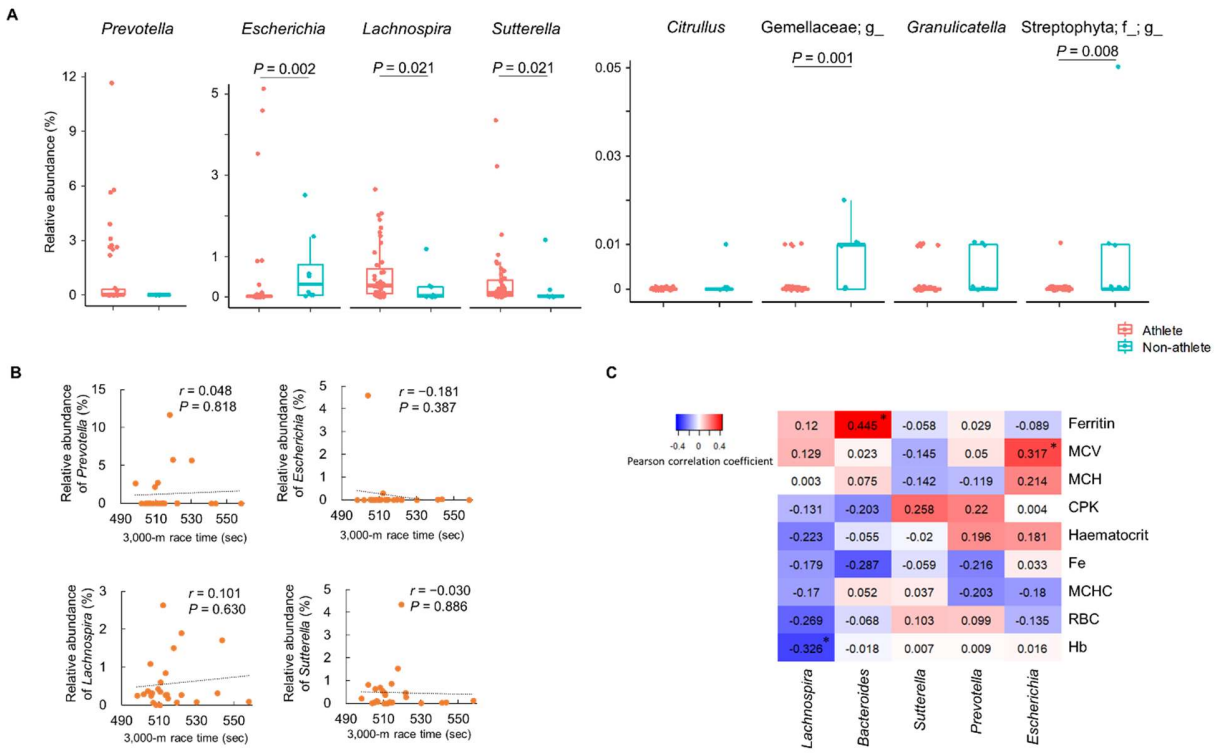
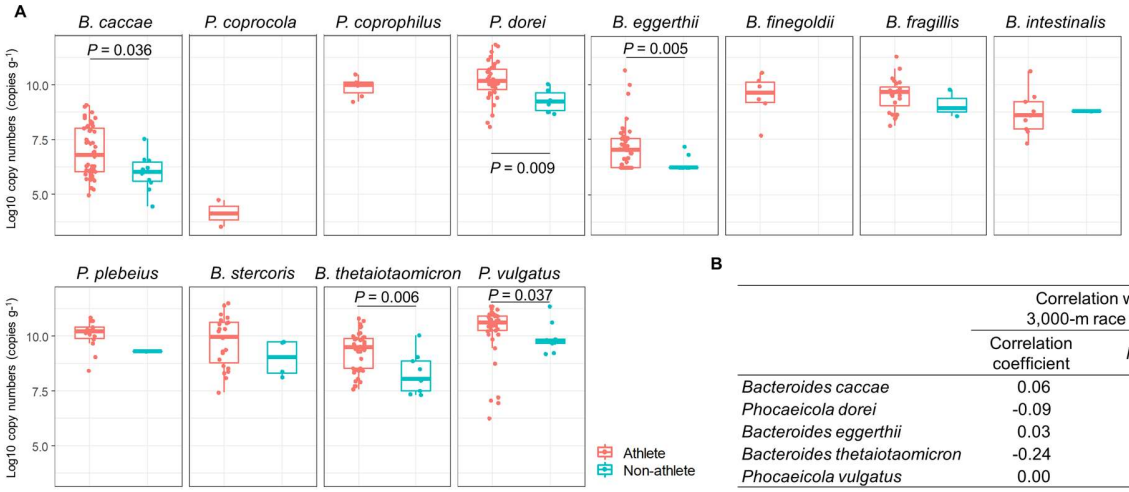


Fig. S1: Relative abundances of *Escherichia*, *Lachnospira,* and *Sutterella* showed differences between the athlete and non-athlete groups but did not correlate with the 3,000-m race time.

A, Box-and-whisker plots of the relative abundances of the genera with | Linear discriminant analysis score | > 2 in the linear discriminant analysis effect size (LEfSe) analysis of the athlete and non-athlete groups. The relative abundance of *Bacteroides* is depicted in Figure 1. The center line represents the median in the box plots, and the box boundaries correspond to the 25th and 75th percentiles. The whiskers represent the most extreme values within 1.5 times the interquartile range below the 25th percentile and above the 75th percentile. The Mann–Whitney *U*-test was used to determine the statistical significance. **B**, Scatterplot of the relative abundances of *Prevotella, Escherichia, Lachnospira,* and *Sutterella* in the athlete group and the 3,000-m race time. The dotted line shows the regression line. Correlations were established using the Pearson correlation coefficient. **C**, Correlations of blood parameters with the relative abundances of *Prevotella*, *Escherichia*, *Lachnospira*, *Sutterella,* and *Bacteroides* in the athlete group. The blood parameters selected in this study are associated with anemia and may affect long-distance

running capacity. The creatine phosphokinase (CPK) levels reflect the degree of muscle injury. Each Pearson correlation coefficient is shown in the heatmap. For all parameters except the 3,000-m race time, the sample size was as follows: athlete group, $n = 43$; non-athlete group, $n = 8$. For the 3,000-m race time, the sample size was as follows: athlete group, $n = 25$. Twenty-three of 48 athletes refused to participate in the measurement of the race time. * $P < 0.05$.
MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; CPK: creatine phosphokinase; MCHC: mean corpuscular hemoglobin concentration; Hb: hemoglobin; RBC: red blood cell.



B

	Correlation with 3,000-m race time	
	Correlation coefficient	*P*-value
Bacteroides caccae	0.06	0.760
Phocaeicola dorei	-0.09	0.698
Bacteroides eggerthii	0.03	0.897
Bacteroides thetaiotaomicron	-0.24	0.280
Phocaeicola vulgatus	0.00	0.990

Fig. S2: Abundance of *B. caccae, P. dorei, B. eggerthii, B. thetaiotaomicron,* and *P. vulgatus* in the athlete group was higher than in the non-athlete group but did not correlate with the 3,000-m race time.

A, Box-and-whisker plots of the log10 copy numbers of 16S rRNA-encoding gene of *Bacteroides* and *Phocaeicola* species determined using species-specific qPCR in the athlete and non-athlete groups. The center line represents the median in the box plots, and the box boundaries represent the 25th and 75th percentiles. The whiskers represent the most extreme values within 1.5 times the interquartile range below the 25th percentile and above the 75th percentile. The Mann–Whitney *U*-test was used for the assessment of statistical significance. **B**, Correlations between the 3,000-m race time and log10 copy numbers of 16S rRNA-encoding gene of the five species with significantly higher copy numbers in the athlete group than in the non-athlete group. Correlations were established using the Pearson correlation coefficient. For the comparison of the copy numbers of the species: athlete group, $n = 48$; non-athlete group, $n = 10$. For the analysis of the correlation with the 3,000-m race time: athlete group, $n = 25$.

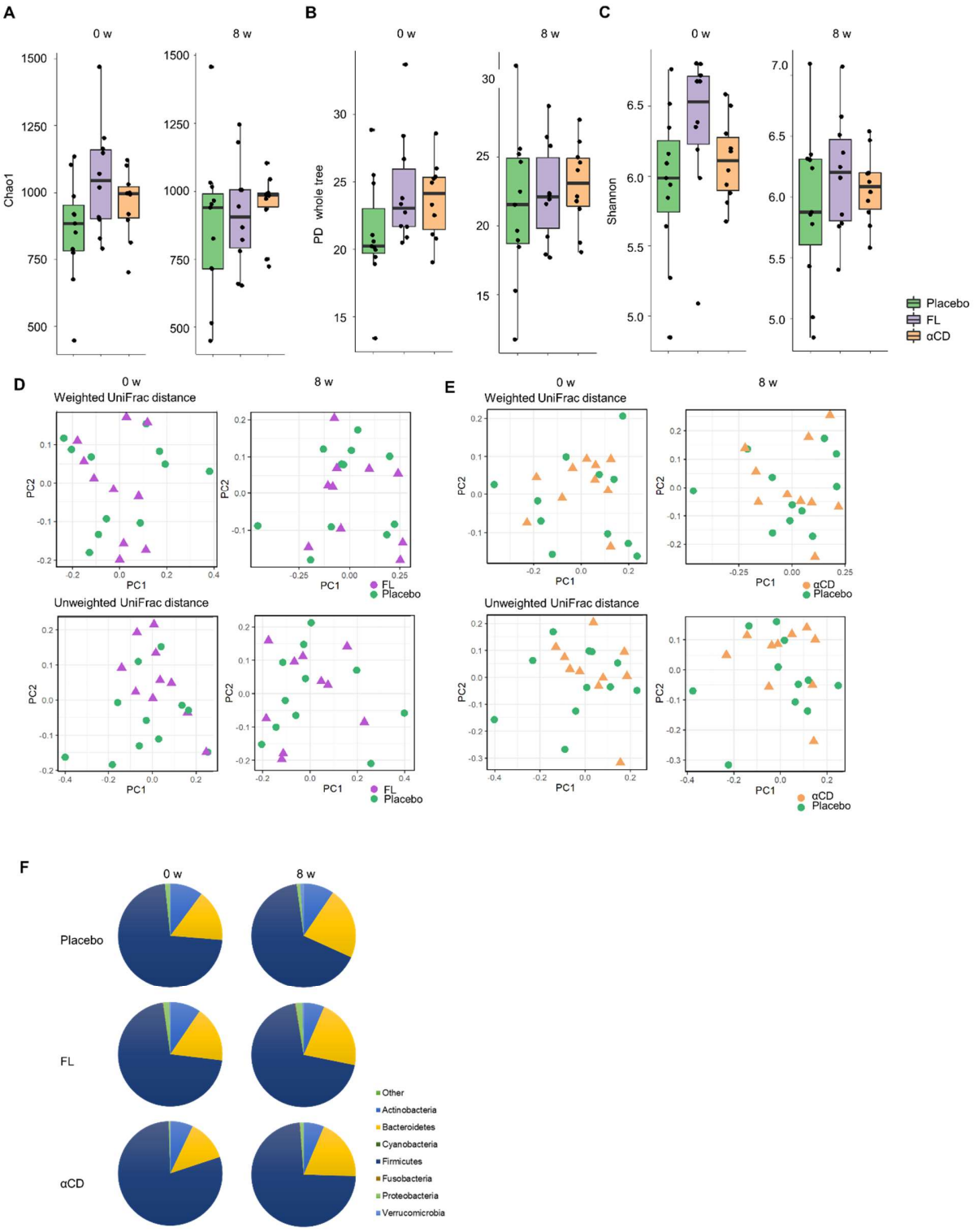

A
0 w
8 w
Chao1
1500
1250
1000
750
500
B
PD_whole_tree
30
25
20
15
C
Shannon
7.0
6.5
6.0
5.5
5.0
Placebo
FL
αCD
D
Weighted UniFrac distance
Unweighted UniFrac distance
PC1
PC2
FL
Placebo
E
αCD
Placebo
F
Placebo
FL
αCD
Other
Actinobacteria
Bacteroidetes
Cyanobacteria
Firmicutes
Fusobacteria
Proteobacteria
Verrucomicrobia

Fig. S3: Supplementation with flaxseed lignans or αCD did not alter the human gut microbiome.

A–**C**, Chao1 estimate (**A**), phylogenetic distance (PD) of the whole tree (**B**), and Shannon diversity index (**C**) of the placebo group, flaxseed lignan (FL) group, and α-cyclodextrin (αCD) groups. The box-and-whisker plots illustrate the distribution of values within each group. In the box plots, the center line represents the median, and the box boundaries represent the 25th and 75th percentiles. The whiskers represent the most extreme values within 1.5 times the interquartile range below the 25th percentile and above the 75th percentile. The statistical significance of the group differences was analyzed using a two-sample *t*-test using Monte Carlo permutations. **D**, **E**, Fecal microbiome profiles of the placebo and FL groups (**D**) and the placebo and αCD groups (**E**). Principal coordinate analysis plots with weighted or unweighted UniFrac distances showed no differences between two treatment groups and placebo. The statistical significance of the differences between the groups was analyzed using permutational multivariate analysis of variance (PERMANOVA). **F**, Phylum-level fecal microbiota composition of the placebo and treated groups. The mean relative abundance of each bacterial phylum is shown in the pie charts. The sample size was as follows: Placebo group, $n = 11$; FL group, $n = 10$; αCD group, $n = 10$.

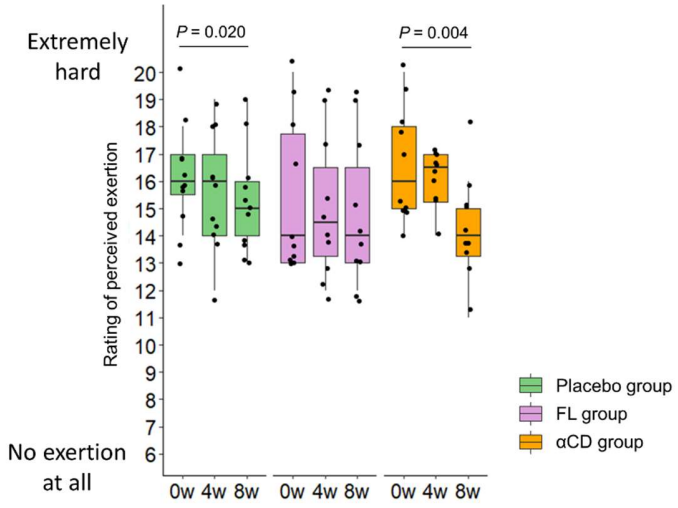


Fig. S4: In the randomized, double-blind, placebo-controlled, parallel-group study, ratings of perceived exertion (RPE) in the αCD and placebo groups were lower after 8 weeks of supplementation than at baseline.

Values of an individual's RPE were measured with Borg's RPE Scale after a 50-min constant-load bike exercise session. The box-and-whisker plots illustrate the distribution of values within each group. In the box plots, the center line represents the median, and the box boundaries represent the 25th and 75th percentiles. The whiskers represent the most extreme values within 1.5 times the interquartile range below the 25th percentile and above the 75th percentile. Statistical significance of the differences between groups (placebo group vs. FL group or αCD group) and within groups (0 weeks vs. 4 or 8 weeks) were analyzed using the Mann–Whitney *U*-test and Wilcoxon signed-rank test, respectively. The sample size was as follows: Placebo group, $n = 11$; FL group, $n = 10$; αCD group, $n = 10$.

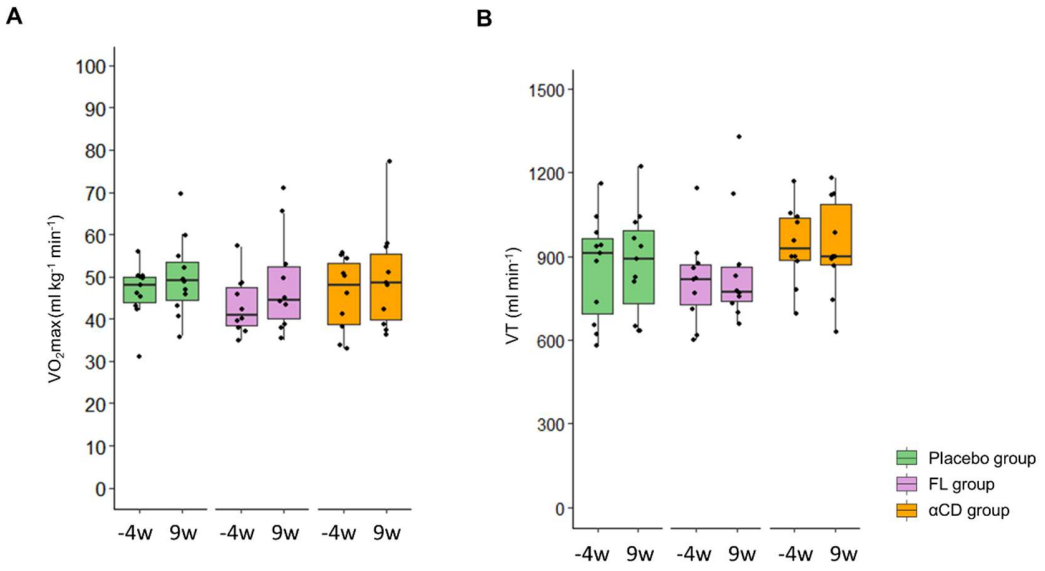


Fig. S5: In the randomized, double-blind, placebo-controlled, parallel-group study, the $\dot{V}O_2$max and ventilatory threshold (VT) showed no significant differences.

$\dot{V}O_2$max (**A**) and VT (**B**) of the participants in placebo, FL, and αCD groups. No significant differences were observed between the placebo and two treated groups, or within groups. The box-and-whisker plots illustrate the distribution of values within each group. In the box plots, the center line represents the median, and the box boundaries represent the 25th and 75th percentiles. The whiskers represent the most extreme values within 1.5 times the interquartile range below the 25th percentile and above the 75th percentile. Statistical significance of the differences between groups (placebo vs. FL or αCD group) and within groups (−4 weeks vs. 9 weeks) was analyzed using an unpaired *t*-test and a paired *t*-test, respectively. The sample size was as follows: Placebo group, $n = 11$; FL group, $n = 10$; αCD group, $n = 10$.

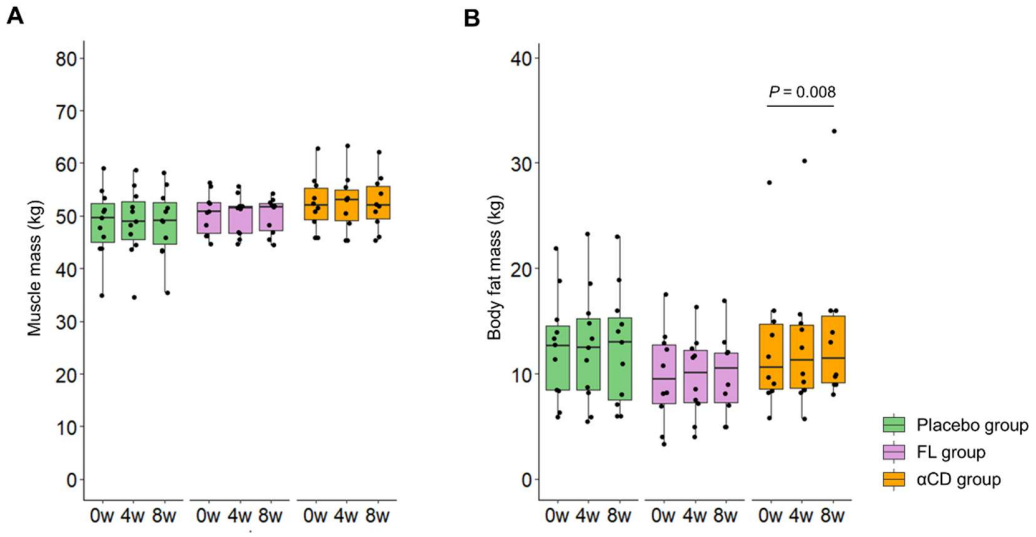
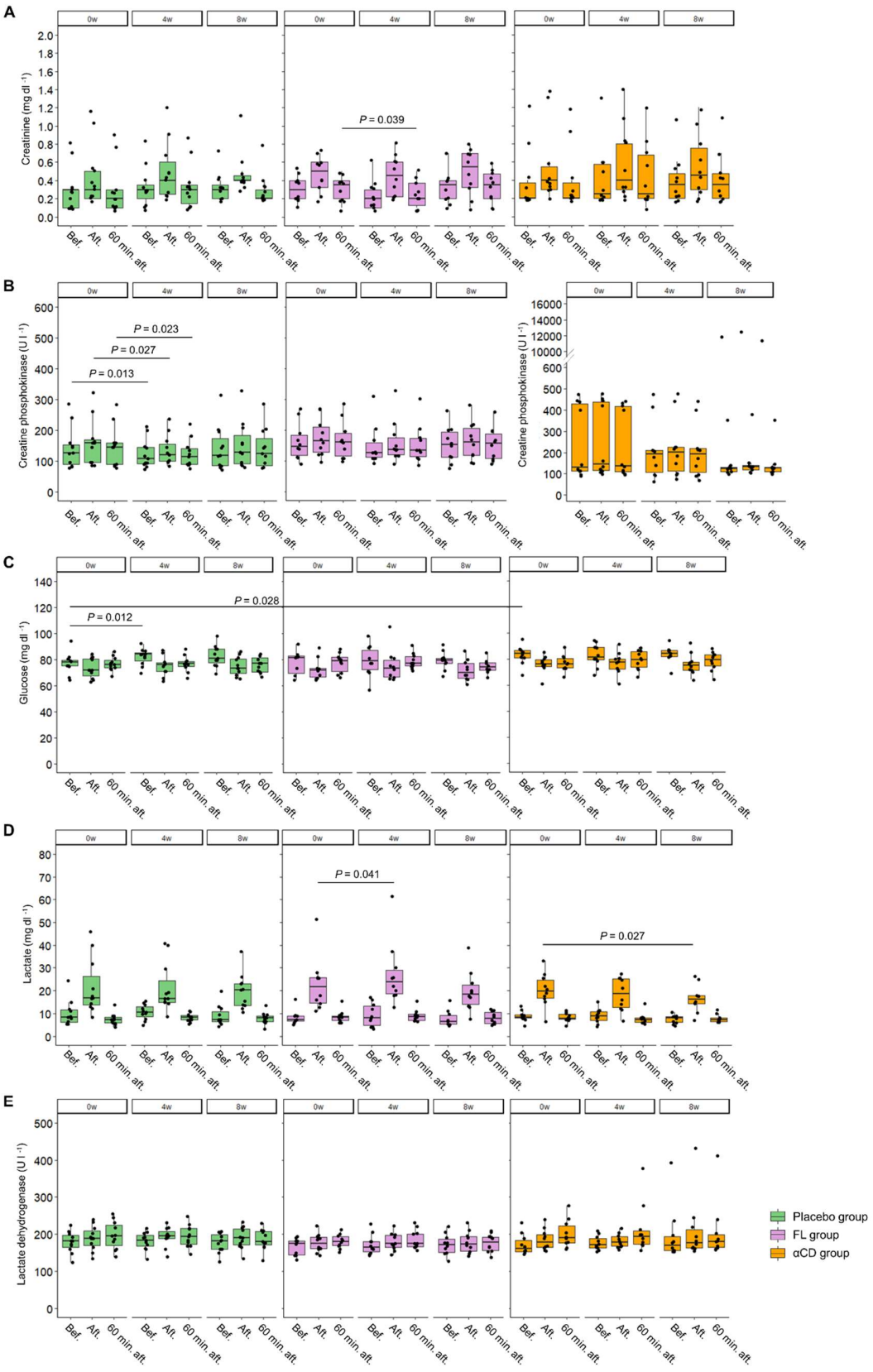


Fig. S6: In the randomized, double-blind, placebo-controlled, parallel-group study, no significant differences in muscle mass were found between baseline (0 weeks) and at 4 and 8 weeks, but body fat mass in the αCD group was slightly higher at 8 weeks than at baseline. Muscle mass (**A**) and body fat mass (**B**) of the participants in the placebo, FL, and αCD groups. The box-and-whisker plots illustrate the distribution of values within each group. In the box plots, the center line represents the median, and the box boundaries represent the 25th and 75th percentiles. The whiskers represent the most extreme values within 1.5 times the interquartile range below the 25th percentile and above the 75th percentile. Statistical significance of the differences in muscle mass between groups (placebo vs. FL or αCD group) and within groups (0 weeks vs. 4 or 8 weeks) was analyzed using an unpaired and a paired *t*-test, respectively. The body fat mass was analyzed using the Mann–Whitney *U*-test and Wilcoxon signed-rank test. The sample size was as follows: Placebo group, $n = 11$; FL group, $n = 10$; αCD group, $n = 10$.


A
Creatinine (mg dl⁻¹)
P = 0.039
B
Creatine phosphokinase (U l⁻¹)
P = 0.023
P = 0.027
P = 0.013
C
Glucose (mg dl⁻¹)
P = 0.028
P = 0.012
D
Lactate (mg dl⁻¹)
P = 0.041
P = 0.027
E
Lactate dehydrogenase (U l⁻¹)
0w
4w
8w
Bef.
Aft.
60 min. aft.
Placebo group
FL group
αCD group

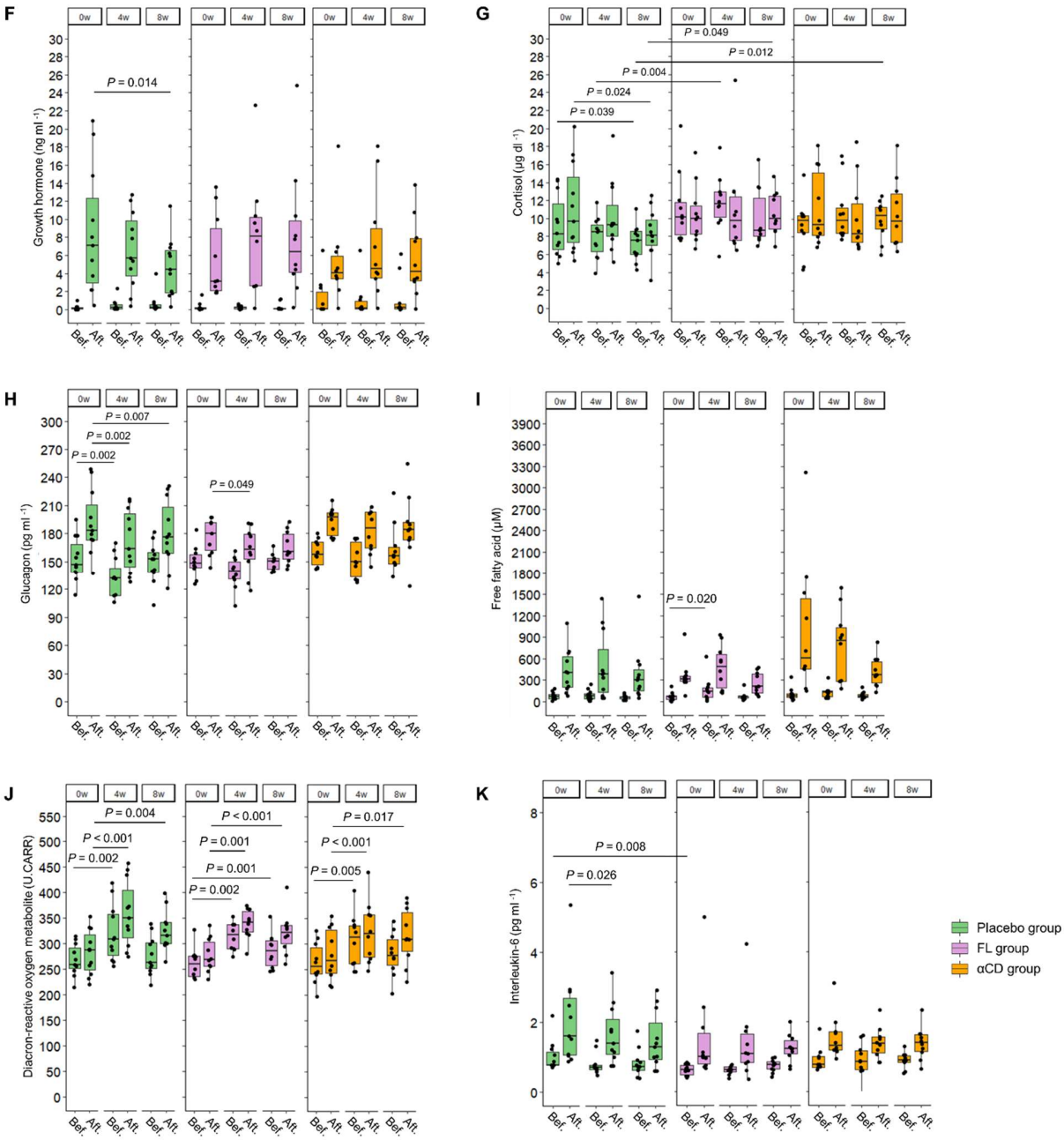
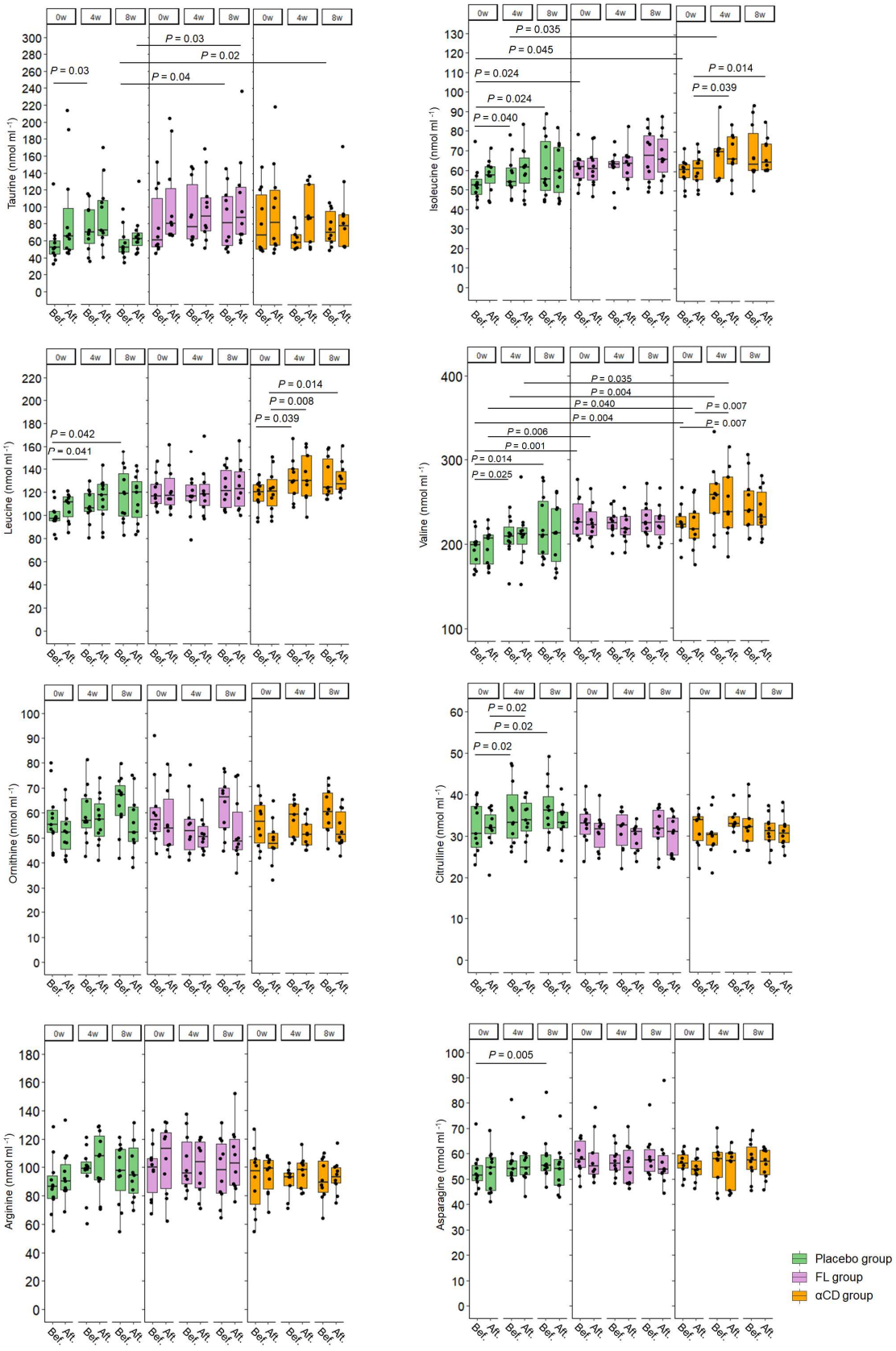
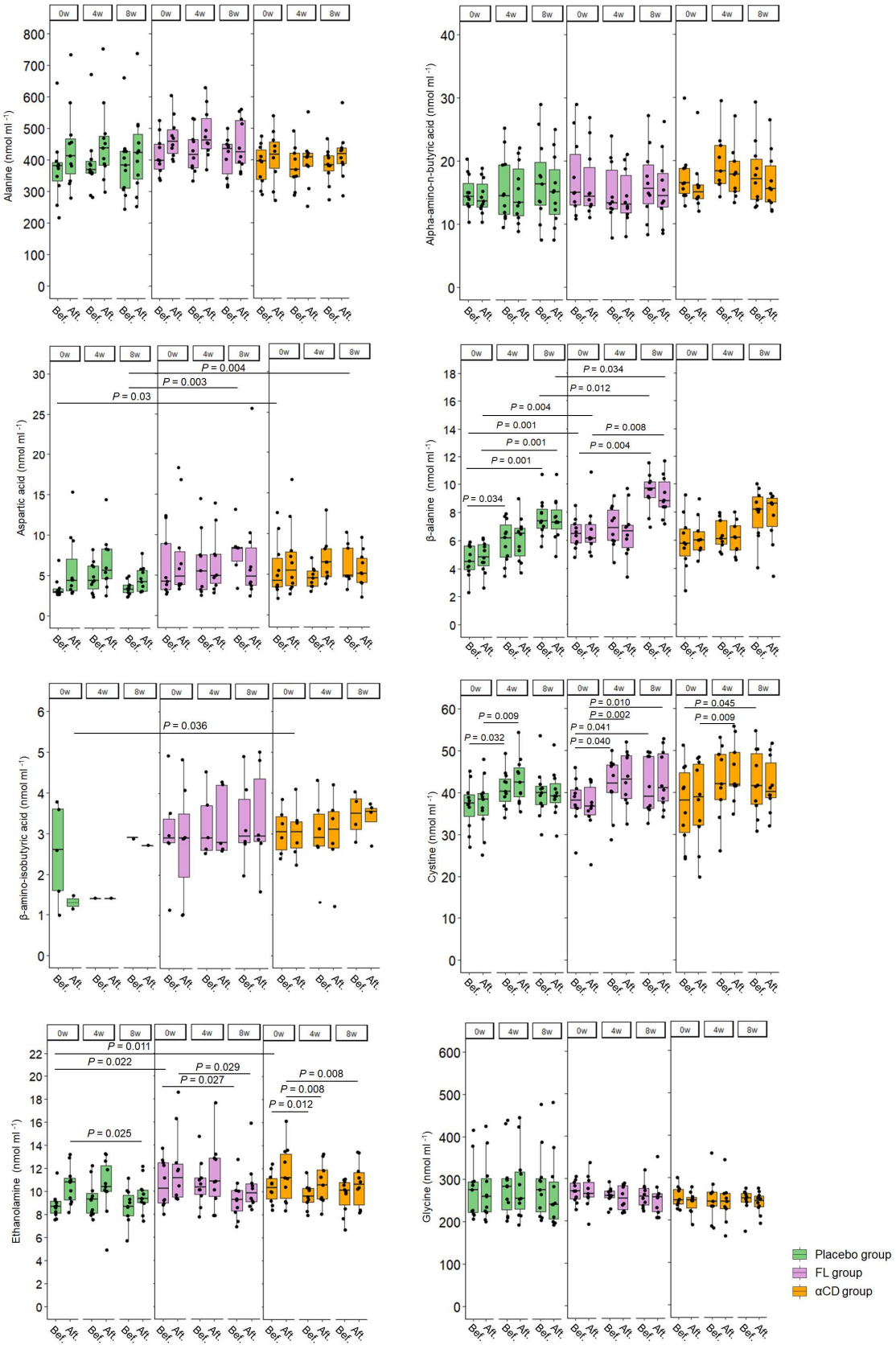


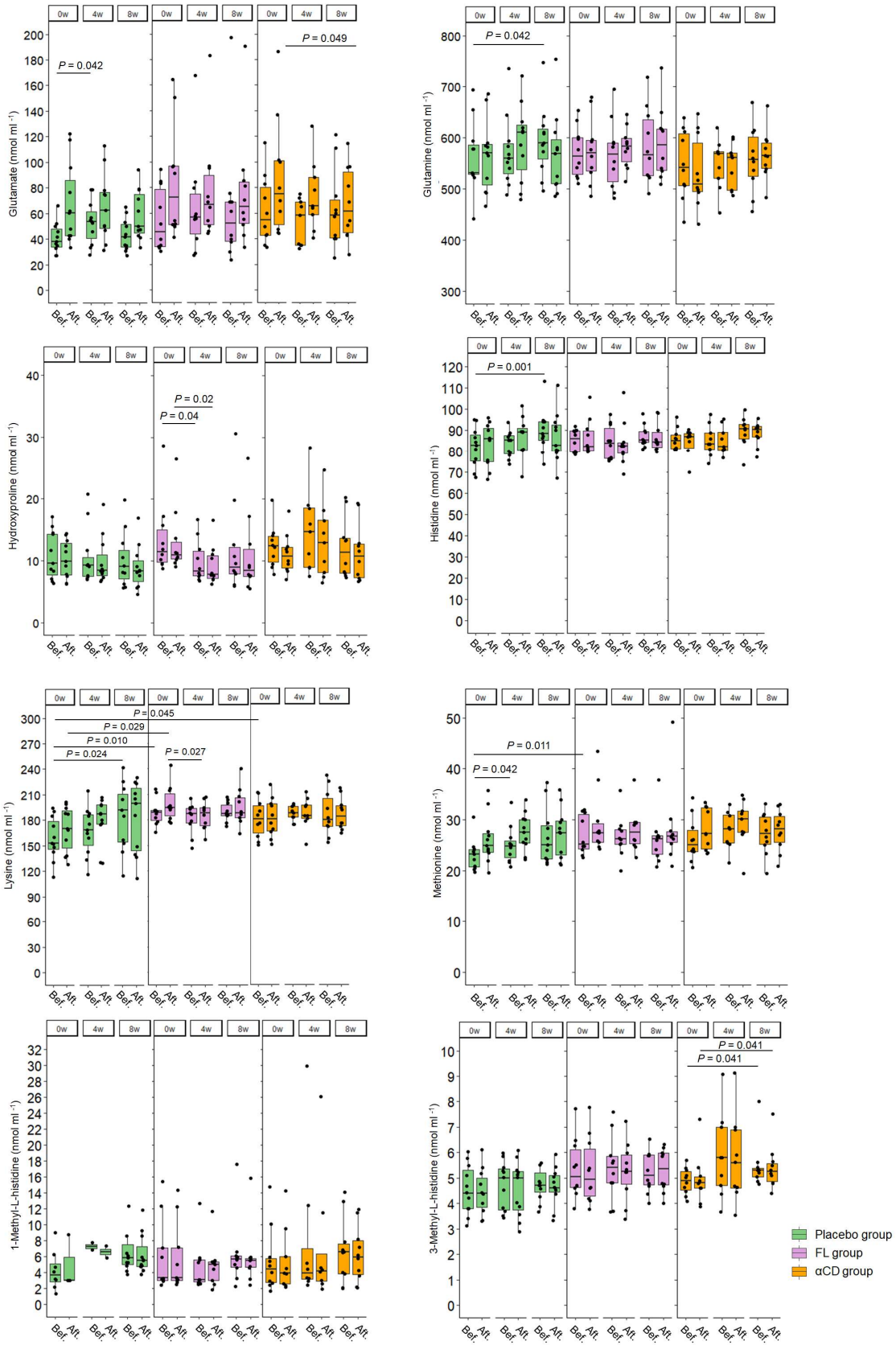
Fig. S7: Hematological parameters at baseline (0 weeks), 4, and 8 weeks in the randomized, double-blind, placebo-controlled, parallel-group study.

The levels of creatinine (**A**), creatine phosphokinase (**B**), glucose (**C**), lactate (**D**), and lactate dehydrogenase (**E**) were measured before and immediately after, and 60 min after 50 min of constant-load exercise performed after 0, 4, and 8 weeks of supplementation. The levels of

hormones (**F–H**), free fatty acids (**I**), diacron-reactive oxygen metabolites (**J**), and interleukin-6 (**K**) were measured before and immediately after the constant-load exercise. The box-and-whisker plots illustrate the distribution of values within each group. In the box plots, the center line represents the median, and the box boundaries represent the 25th and 75th percentiles. The whiskers represent the most extreme values within 1.5 times the interquartile range below the 25th percentile and above the 75th percentile. Statistical significance of the differences between groups (placebo vs. FL or αCD group) and within groups (0 weeks vs. 4 or 8 weeks) was analyzed using the Mann–Whitney *U*-test and Wilcoxon signed-rank test, respectively. The sample size was as follows: Placebo group, $n = 11$; FL group, $n = 10$; αCD group, $n = 10$. Bef.: before 50 min of constant-load exercise; Aft.: immediately after 50 min of constant-load exercise; 60 min aft.: 60 min after 50 min of constant-load exercise.


0w
4w
8w
Bef.
Aft.
Taurine (nmol ml⁻¹)
P = 0.03
P = 0.03
P = 0.02
P = 0.04
Isoleucine (nmol ml⁻¹)
P = 0.035
P = 0.045
P = 0.024
P = 0.024
P = 0.040
P = 0.014
P = 0.039
Leucine (nmol ml⁻¹)
P = 0.042
P = 0.041
P = 0.014
P = 0.008
P = 0.039
Valine (nmol ml⁻¹)
P = 0.035
P = 0.004
P = 0.040
P = 0.004
P = 0.007
P = 0.007
P = 0.006
P = 0.001
P = 0.014
P = 0.025
Ornithine (nmol ml⁻¹)
Citrulline (nmol ml⁻¹)
P = 0.02
P = 0.02
P = 0.02
Arginine (nmol ml⁻¹)
Asparagine (nmol ml⁻¹)
P = 0.005
Placebo group
FL group
αCD group


Alanine (nmol ml⁻¹)
Alpha-amino-n-butyric acid (nmol ml⁻¹)
Aspartic acid (nmol ml⁻¹)
β-alanine (nmol ml⁻¹)
β-amino-isobutyric acid (nmol ml⁻¹)
Cystine (nmol ml⁻¹)
Ethanolamine (nmol ml⁻¹)
Glycine (nmol ml⁻¹)
0w
4w
8w
Bef.
Aft.
P = 0.03
P = 0.003
P = 0.004
P = 0.034
P = 0.012
P = 0.001
P = 0.008
P = 0.036
P = 0.009
P = 0.032
P = 0.010
P = 0.002
P = 0.041
P = 0.040
P = 0.045
P = 0.011
P = 0.022
P = 0.029
P = 0.027
P = 0.025
P = 0.012
Placebo group
FL group
αCD group


0w
4w
8w
Bef.
Aft.
Glutamate (nmol ml⁻¹)
P = 0.042
P = 0.049
Glutamine (nmol ml⁻¹)
P = 0.042
Hydroxyproline (nmol ml⁻¹)
P = 0.02
P = 0.04
Histidine (nmol ml⁻¹)
P = 0.001
Lysine (nmol ml⁻¹)
P = 0.045
P = 0.029
P = 0.010
P = 0.024
P = 0.027
Methionine (nmol ml⁻¹)
P = 0.011
P = 0.042
1-Methyl-L-histidine (nmol ml⁻¹)
3-Methyl-L-histidine (nmol ml⁻¹)
P = 0.041
P = 0.041
Placebo group
FL group
αCD group

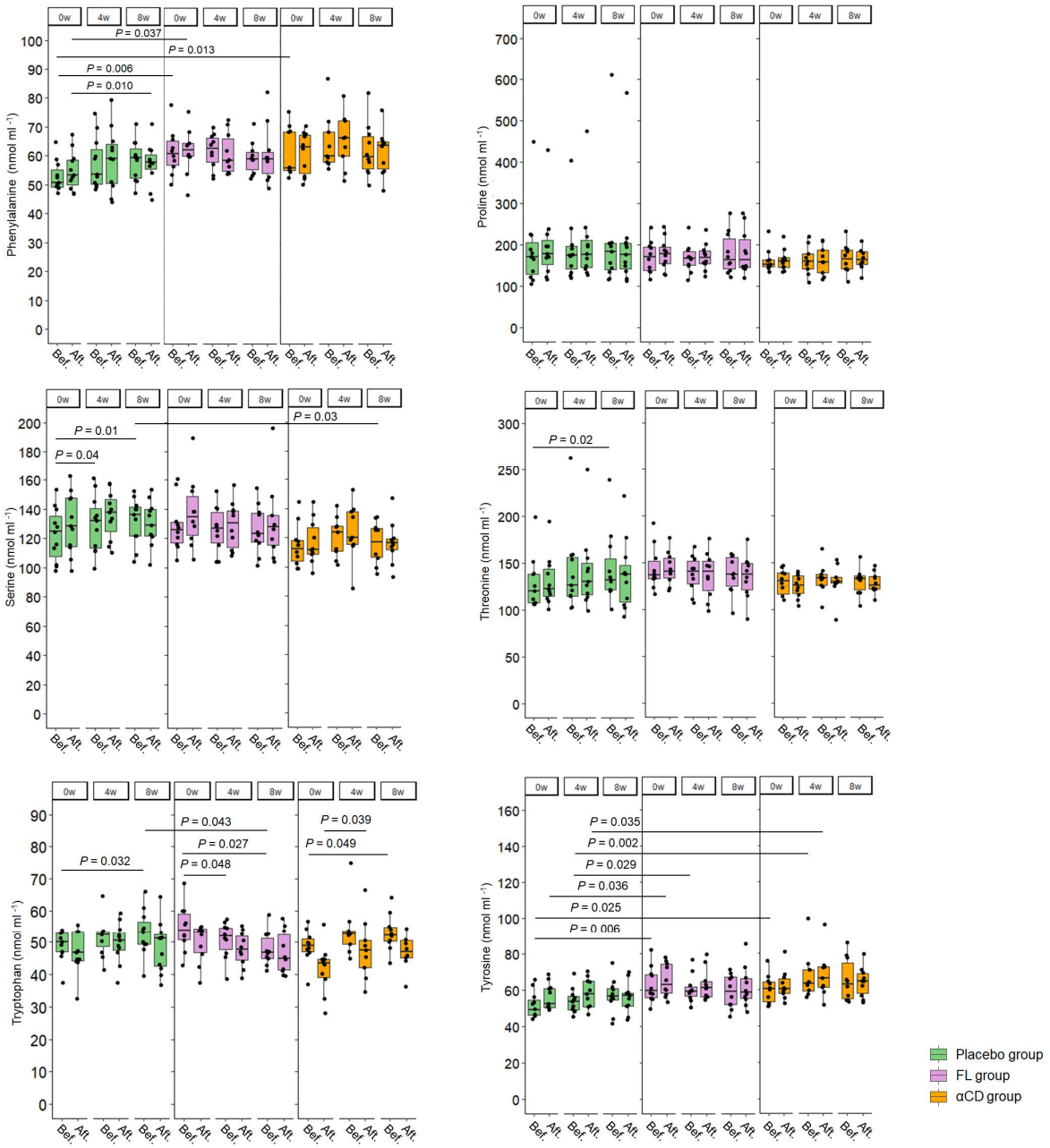


Fig. S8: Blood amino acid levels at baseline (0 weeks), 4 weeks, and 8 weeks in the randomized, double-blind, placebo-controlled, parallel-group study.

Blood amino acid levels were measured before and immediately after 50 min of constant-load exercise performed after 0, 4, and 8 weeks of supplementation. The box-and-whisker plots illustrate the distribution of values within each group. In the box plots, the center line represents

the median, and the box boundaries represent the 25th and 75th percentiles. The whiskers represent the most extreme values within 1.5 times the interquartile range below the 25th percentile and above the 75th percentile. Statistical significance of the differences between groups (placebo vs. FL or αCD group) and within groups (0 weeks vs. 4 or 8 weeks) was analyzed using an unpaired *t*-test or the Mann–Whitney *U*-test, and a paired *t*-test or the Wilcoxon signed-rank test, respectively. The statistical tests were selected based on the outcomes of the normality test using the Shapiro–Wilk test. The sample size was as follows: Placebo group, $n = 11$; FL group, $n = 10$; αCD group, $n = 10$.

Bef.: before 50 min of constant-load exercise; Aft.: immediately after 50 min of constant-load exercise.

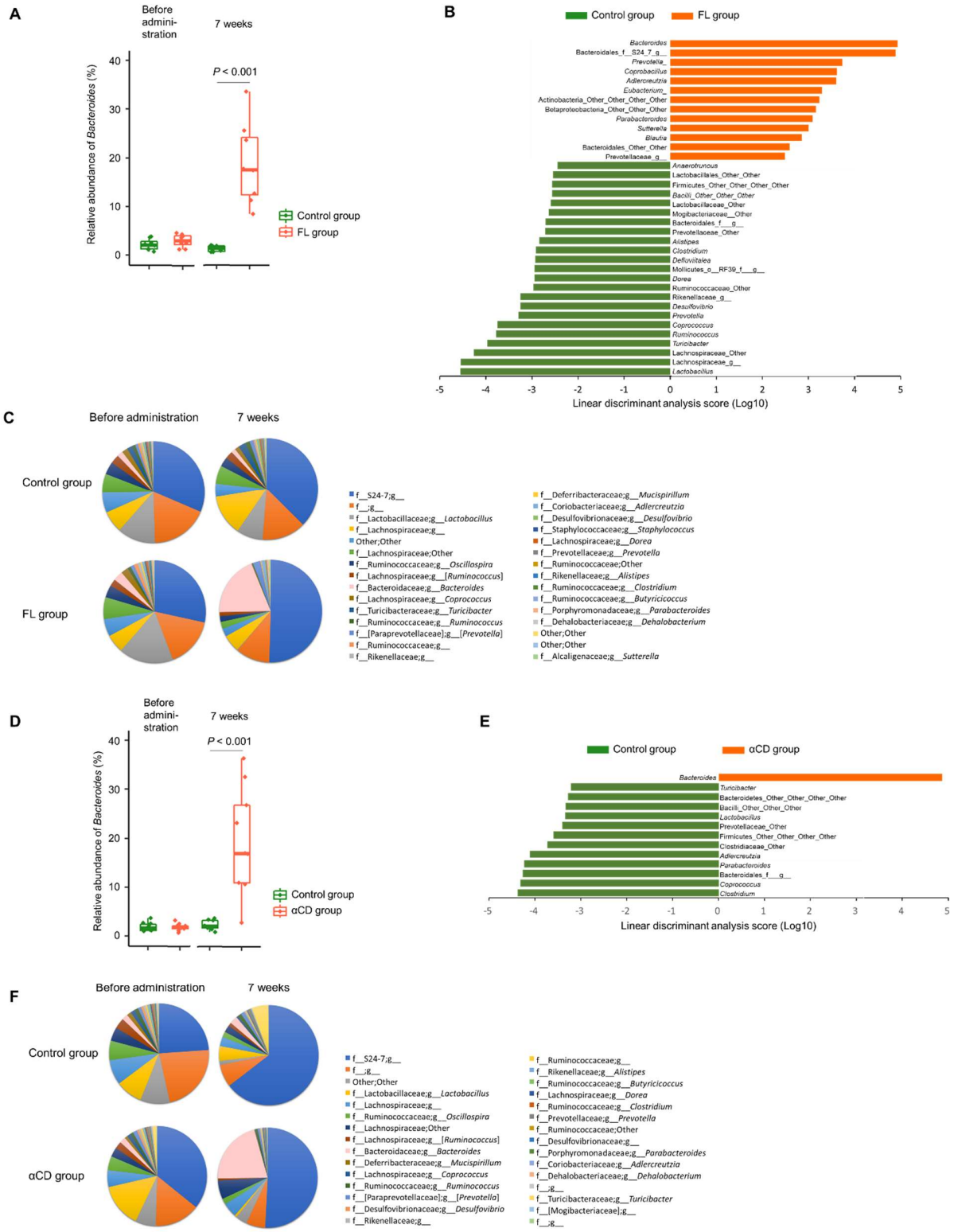


Fig. S9: Relative abundance of the genus *Bacteroides* was increased in the FL- and αCD-fed mice, with αCD increasing the abundance of *Bacteroides* specifically.

A, **D**, Box-and-whisker plots showing the relative abundance of *Bacteroides* in the groups fed a diet containing 5% (w/w) FL or α-CD and their respective control diet-fed groups as determined by 16S rRNA-encoding gene amplicon sequencing with the MiSeq platform (n = 8 for the FL group and associated control group, n = 10 for the αCD group and associated control group). In the box plots, the center line represents the median, and the box boundaries represent the 25th and 75th percentiles. The whiskers represent the most extreme values within 1.5 times the interquartile range below the 25th percentile and above the 75th percentile. The Mann–Whitney *U*-test was used for the statistical analysis. **B**, **E**, LEfSe analysis comparing the FL or αCD group with the associated control group using genus-level microbial composition data. All genera with | Linear discriminant analysis score | > 2 in each group are displayed in this figure. **C**, **F**, Genus-level fecal microbiota composition of the FL or αCD group with the associated control group. The mean relative abundance of each bacterial genus is shown in the pie charts.

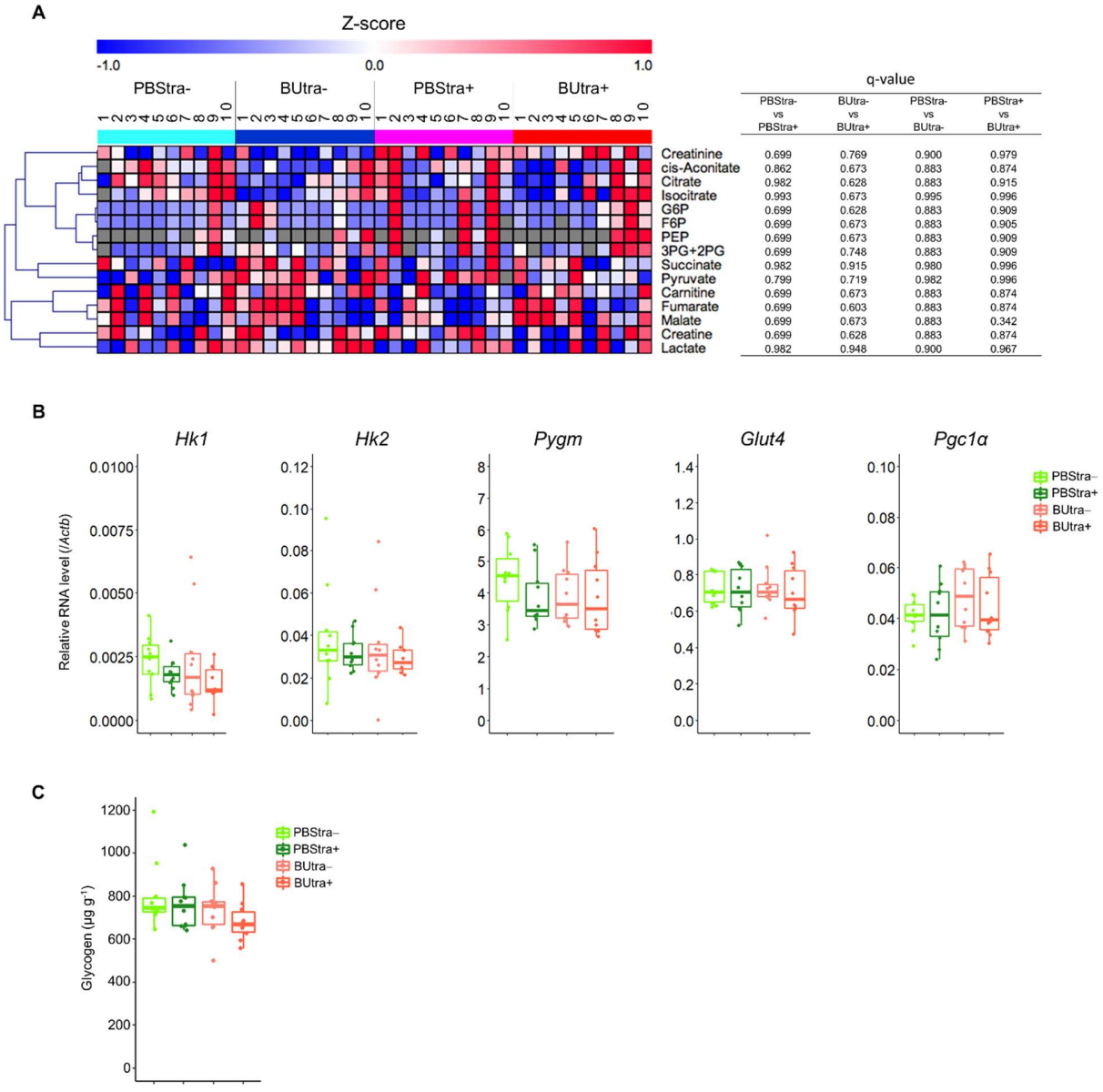


Fig. S10: Metabolite concentrations, mRNA levels related to energy acquisition, and glycogen content in murine muscle were comparable among the four groups.

A, The metabolite concentrations related to energy acquisition in murine quadriceps femoris samples from the PBStra− (n = 10), PBStra+ (n = 10), BUtra− (n = 10), and BUtra+ (n = 10) groups, determined using capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS). A heatmap depicts the Z-scores of metabolites involved in energy metabolism, which were calculated from the quantitative values. Individual mice are represented as columns. The q-

value of each metabolite between each group is shown on the right side. Statistical significance of the differences between groups was analyzed using Student's *t*-tests with Benjamini–Hochberg false discovery rate correction. **B**, Box-and-whisker plots of the mRNA levels of four genes related to glycolysis (*Hk1*, *Hk2*, *Pygm*, and *Glut4*) and *Pgc1α* in the quadriceps femoris samples of the PBStra− (n = 10), PBStra+ (n = 10), BUtra− (n = 10), and BUtra+ (n = 10) mice measured by reverse transcription qPCR. **C**, Glycogen content in the murine quadriceps femoris samples from the PBStra− (n = 10), PBStra+ (n = 10), BUtra− (n = 10), and BUtra+ (n = 10) groups. 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. Statistical significance of the differences among groups was analyzed using the Tukey–Kramer test (*HK2*, *Pygm*, and *Pgc1*α) or Steel–Dwass test (*Hk1*, *Glut4*, and glycogen content). The statistical tests were selected based on the outcomes of the normality test using the Shapiro–Wilk test. PBStra+: PBS-administered mice subjected to exercise; PBStra−: PBS-administered mice not subjected to exercise; BUtra+: *B. uniformis*-administered mice subjected to exercise; BUtra−: *B. uniformis*-administered mice not subjected to exercise; G6P: glucose-6-phosphate; F6P: fructose 6-phosphate; PEP: phosphoenolpyruvate; 3PG: 3-phosphoglycerate; 2PG: 2-phosphoglycerate.

Table S1. Baseline data of participants included in the randomized, double-blind, placebo-controlled, parallel-group human study with FL or αCD as test supplements.

	Placebo (n = 11)	FL group (n = 10)	αCD group (n = 10)	P-value [1)]	
				Placebo vs. FL group	Placebo vs. αCD group
Age (years)	36.3 ± 9.6	33.9 ± 10.0	34.5 ± 10.9	0.59	0.70
Weight (kg)	64.01 ± 9.68	63.12 ± 6.34	67.76 ± 7.54	0.81	0.34
Body mass index (kg/m^2)	22.01 ± 2.43	21.53 ± 1.67	22.23 ± 2.43	0.61	0.84
Heart rate (bpm)	72.5 ± 9.8	70.5 ± 13.0	69.1 ± 9.4	0.70	0.43
VO_2max (mL/kg/min)	46.40 ± 6.45	43.20 ± 6.83	45.67 ± 8.66	0.28	0.83

These data were collected before supplementation.
Mean ± standard deviation
[1] Two-tailed unpaired *t*-test

Table S2. Primers and probes used for quantitative real-time PCR for *Bacteroides* and *Phocaeicola* species.

Target	Primer name	Sequence (5'-3')	Probe name	Sequence (5'Fam-3'Tam)	Reference
B. caccae	B.cac-TaqMan-F	AAACCCATACGCCGCAAG	B.cac-TaqMan-Prb	TGTGAAGGTGCTGCATGGTTGTCGT	49
	B.cac-TaqMan-R	GACACCTCACGGCACGAG			
P. coprocola	BaCOP-F	TATGGTGAGATTGCATGATGG	-		50
	BaCOP-R	ATGAACGTCAGTTACAGTTTAGCAA	-		
P. coprophilus	BaCPP-F	GGGTTGTAAACTTCTTTTGTGC	-		50
	BaCPP-R	GCCTCAACCGTACTCAAGGT	-		
P. dorei	BaDOR-F	GGAAACGGTTCAGCTAGCAATA	-		50
	BaDOR-R	AGTCTTGTCAGAGTCCTCAGCATC	-		
B. eggerthii	B.egg-TaqMan-F	CCCGATAGTATAGTTTTTCCGC	B.egg-TaqMan-Prb	TTCGGTTATCGATGGGGATGCGTTC	49
	B.egg-TaqMan-R	TCCTCTCAGAACCCCTATCCAT			
B. finegoldii	BaFIN-F	CCGGATGGCATAGGATTGTC	-		50
	BaFIN-R	CGTAGGAGTTTGGACCGTGT	-		
B. fragilis	FW3	AGGATTCCGGTAAAGGATGG	-		This study
	RV3	GTTCAGGCTAGCGCCCATT	-		
B. intestinalis	BaINT-F	AGCATGACCTAGCAATAGGTTG	-		50
	BaINT-R	ACGCATCCCCATCGATTAT	-		
P. plebeius	BaPLE-F	ATCATTAAAGATTTATCGGTGTACG	-		50
	BaPLE-R	ACTTTCACAGCTGACTTAACGAC	-		
B. stercoris	B.ste-TaqMan-F	GCTTGCTTTGATGGATGGC	B.ste-TaqMan-Prb	CCAACCTGCCGACAACACTGGGATA	49
	B.ste-TaqMan-R	CATGCGGGAAAACTATGCC	-		
B. thetaiotaomicron	B.the-TaqMan-F	GCAAACTGGAGATGGCGA	B.the-TaqMan-Pro	TCGATGGGGATGCGTTCCATTAGG	49
	B.the-TaqMan-R	AAGGTTTGGTGAGCCGTTA	-		
B. uniformis	B.uni-TaqMan-F	TCTTCCGCATGGTAGAACTATTA	B.uni-TaqMan-Prb	CGTTCCATTAGGTTGTTGGCGGGG	49
	B.uni-TaqMan-R	ACCGTGTCTCAGTTCCAATGTG	-		
P. vulgatus	B.vul-TaqMan-F	CGGGCTTAAATTGCAGATGA	B.vul-TaqMan-Prb	TGAAAGCCGTAAGCCGCAAGG	49
	B.vul-TaqMan-R	CATGCAGCACCTTCACAGAT	-		

The TaqMan probes were labeled with the fluorescent dye 6-carboxyfluorescein (FAM) at the 5' end and 6-carboxytetramethylrhodamine (TAMRA) at the 3' end.

Table S3. Composition of supplements (daily dose) used in the randomized, double-blind, placebo-controlled, parallel-group human study with FL or αCD as test supplements.

	FL supplement (mg)	αCD supplement (mg)	Placebo (mg)
FL [1)]	200.1	0.0	0.0
αCD [2)]	0.0	200.1	0.0
Maltitol	487.1	487.1	687.2
Tricalcium phosphate	19.4	19.4	19.4
Hydroxypropyl cellulose	9.0	9.0	9.0
Calcium stearate	30.0	30.0	30.0
Starch	3.0	3.0	3.0
Silicon dioxide	1.5	1.5	1.5

[1] NIPPN flaxseed lignans (Nippon Flour Mills, Tokyo, Japan)
[2] Dexypearl-α (Ensuiko Sugar Refining, Tokyo, Japan)