Appendix 1

Method of targeted LC-MS/MS analysis

Chemicals and reagents

Standards obtained from Sigma-Aldrich (St. Louis, MO, USA), Steraloids Inc., (Newport, RI, USA) and TRC Chemicals (Toronto, ON, Canada) were used to make a 5-mg/mL stock solution, using which stock calibration solutions were prepared. Analytical grade formic acid was obtained from Sigma-Aldrich. Methanol (Optima LC-MS), acetonitrile (Optima LC-MS), and isopropanol (Optima LC-MS) were all purchased from Thermo-Fisher Scientific (FairLawn, NJ, USA). Ultrapure water was produced by a Mill-Q Reference system equipped with a liquid chromatography-mass spectrometry (LC-MS) Pak filter (Millipore, Billerica, MA, USA).

Targeted metabolomics analysis

Targeted metabolomics analysis on plasma samples were conducted using the Q300 Metabolite Assay Kit (Human Metabolomics Institute, Inc., Shenzhen, China) based on the method previously published (23) with modifications. In brief, 25 µL of plasma or fecal extract was added to a 96-well plate and transferred to the Biomek 4000 workstation (Biomek 4000, Beckman Coulter, Inc., Brea, CA, USA). Ice-cold methanol with partial internal standards was automatically added to each sample and vortexed vigorously for 5 min. After centrifuging at 4,000 g for 30 min (Allegra X-15R, Beckman Coulter, Inc., Indianapolis, IN, USA), 30 µL of supernatant was transferred to a clean 96-well plate and 20 µL of freshly prepared derivative reagent was added to each well. Derivatization was carried out for 60 min at 30 ℃ in a sealed plate. After derivatization, 350 µL of ice-cold 50% methanol solution was added to dilute the sample. The plate was then stored at –20 ℃ for 20 min and centrifuged at 4,000 g for 30 min at 4 ℃. Next, 135 µL of supernatant was transferred to a new 96-well plate with 15 µL internal standards in each well. Serial dilutions of the derivatized stock standards were added to the remaining wells. Finally, the plate was sealed for UPLC-MS analysis.

Instrumentation

An ultra-performance liquid chromatography coupled to tandem mass spectrometry (ACQUITY UPLC-Xevo TQ-S, Waters Corp., Milford, MA, USA) was used to quantitatively measure the metabolites. The entire LC-MS system was controlled by MassLynx 4.1 software. All chromatographic separations were performed with an ACQUITY BEH C18 column (1.7 µm, 100 mm × 2.1 mm internal dimensions) (Waters Corp.).

Data analysis

The raw data files generated by UPLC-MS/MS were processed using the TMBQ software (v1.0, Human Metabolomics Institute, Inc.) to perform peak integration, calibration, and quantitation for each metabolite. The current TMBQ is hosted on Dell PowerEdge R540 Servers operated with RedHat Enterprise Linux 7.5. The secured Java UI (UserInterface) permits the user have access to use a great variety of statistical tools for viewing and exploring project data on its own desire.

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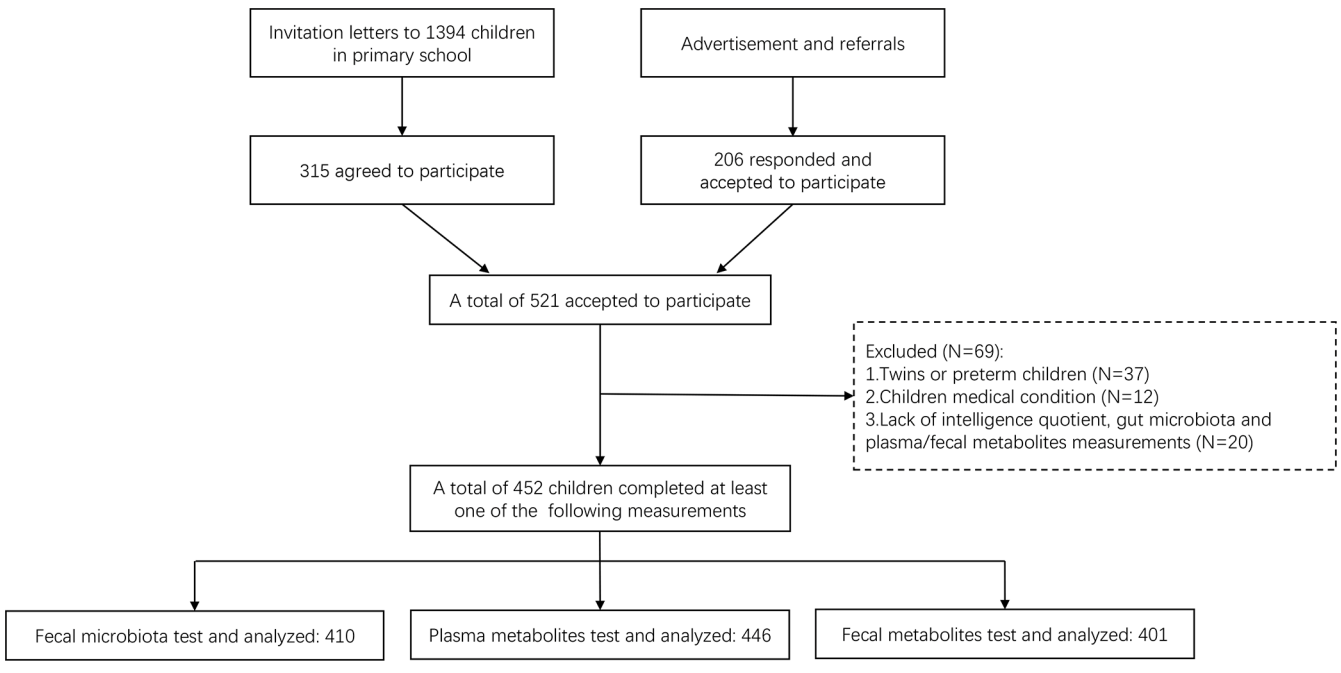

Figure S1 Research recruitment flow chart and the sample size of children completed microbiota/metabolites tests.

Table S1 Explained variance of covariates phenotypes by variation in the microbiota and metabolite

Phenotype	V(O)/Vp (%)	SE (%)	P
Microbiota at genus level			
Age	2.49	3.42	0.149
Sex	5.17	3.50	0.004*
Energy intake	1.40	2.32	0.193
MET	10.67	5.84	0.007*
Fiber intake	2.19	3.08	0.201
Delivery mode	0.00	3.05	0.500
Household income	3.99	3.59	0.061
Maternal education level	18.59	6.50	<0.001*
Paternal education level	5.02	4.28	0.063
Use of calcium supplement	0.00	1.74	0.500
Use of multivitamin supplement	0.85	2.35	0.346
Microbiota at species level			
Age	4.85	3.76	0.056
Sex	6.17	3.93	0.025*
Energy intake	0.00	3.04	0.500
MET	2.37	3.28	0.199
Fiber intake	9.07	4.89	0.028*
Delivery mode	0.00	3.17	0.500
Household income	1.17	2.84	0.313
Maternal education level	2.35	3.28	0.193
Paternal education level	5.89	4.09	0.050
Use of calcium supplement	4.16	3.70	0.084
Use of multivitamin supplement	3.65	4.17	0.214
Alpha-diversity			
Age	0.14	0.57	0.367
Sex	1.33	1.99	0.125
Energy intake	0.10	0.53	0.411
MET	1.10	1.69	0.135
Fiber intake	4.60	5.57	0.008*
Delivery mode	0.13	0.58	0.383
Household income	0.00	0.95	0.500
Maternal education level	0.00	0.72	0.500
Paternal education level	0.07	0.56	0.450
Use of calcium supplement	0.00	2.05	0.500
Use of multivitamin supplement	0.00	0.86	0.500
Plasma metabolite[†]			
Age	26.70	7.02	<0.001*
Sex	38.86	6.72	<0.001*
Energy intake	8.67	4.74	0.005*
MET	4.29	4.55	0.310
Fiber intake	0.32	1.71	0.425
Delivery mode	0.00	1.56	0.500
Household income	12.60	5.46	0.002*
Maternal education level	23.72	6.35	<0.001*
Paternal education level	30.06	6.72	<0.001*
Use of calcium supplement	3.14	3.99	0.280
Use of multivitamin supplement	0.29	1.62	0.420
Fecal metabolite			
Age	2.40	3.78	0.288
Sex	0.00	3.43	0.500
Energy intake	2.71	3.87	0.344
MET	0.00	3.19	0.500
Fiber intake	2.03	2.65	0.123
Delivery mode	1.08	2.24	0.255
Household income	0.00	1.77	0.500
Maternal education level	3.44	4.33	0.217
Paternal education level	0.00	2.88	0.500
Use of calcium supplement	0.67	2.43	0.419
Use of multivitamin supplement	1.67	3.07	0.321

Explained variance estimated using REML models. Physical activity level is represented by MET. *, P<0.05. [†], n=444 in model of plasma metabolites. V(O)/Vp, explained variance; SE, standard error; MET, metabolic equivalent; REML, restricted maximum likelihood.

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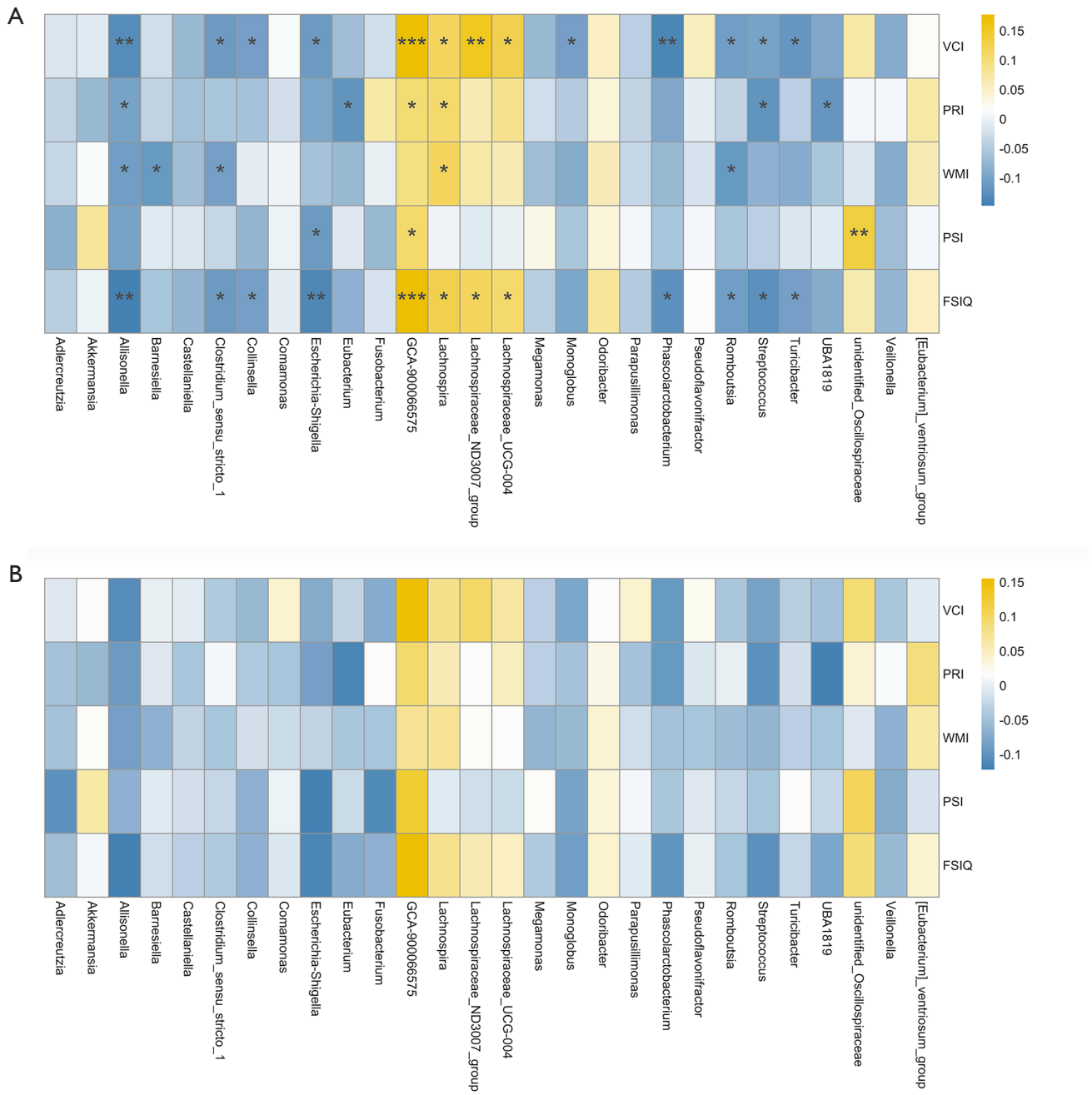

Figure S2 Spearman correlation and multiple linear regression analysis of the association of intelligence scores with selected microbiota counts at genus level. (A) Spearman correlation analysis of the association of intelligence scores with selected microbiota counts at genus level. (B) Multiple linear regression analysis of intelligence scores with selected microbiota counts at genus level after adjustment for age, sex, maternal education level, physical activity level and correction for multiple testing. The Spearman's rank correlation coefficient and β coefficient are indicated by the color of the cells. Positive or negative correlations and β coefficient are represented by shades of yellow or blue respectively. *, P<0.05; **, P<0.01; ***, P<0.001. VCI, Verbal Comprehension Index; PRI, Perceptual Reasoning Index; WMI, Working Memory Index; PSI, Processing Speed Index; FSIQ, Full-Scale Intelligence Quotient.

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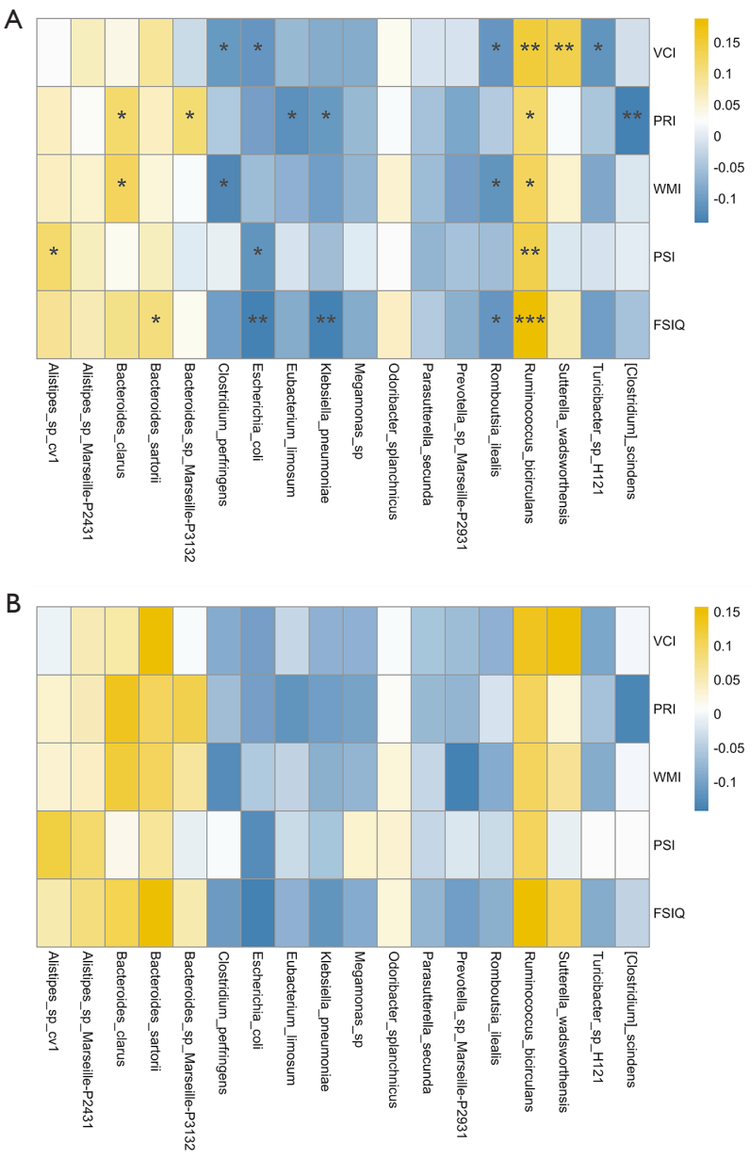

Figure S3 Spearman correlation and multiple linear regression analysis of the association of intelligence scores with selected microbiota counts at species level. (A) Spearman correlation analysis of the association of intelligence scores with selected microbiota counts at species level. (B) Multiple linear regression analysis of intelligence scores with selected microbiota counts at species level after adjustment for age, sex, fiber intake and correction for multiple testing. The Spearman's rank correlation coefficient and β coefficient are indicated by the color of the cells. Positive or negative correlations and β coefficient are represented by shades of yellow or blue respectively. *, P<0.05; **, P<0.01; ***, P<0.001. VCI, Verbal Comprehension Index; PRI, Perceptual Reasoning Index; WMI, Working Memory Index; PSI, Processing Speed Index; FSIQ, Full-Scale Intelligence Quotient.

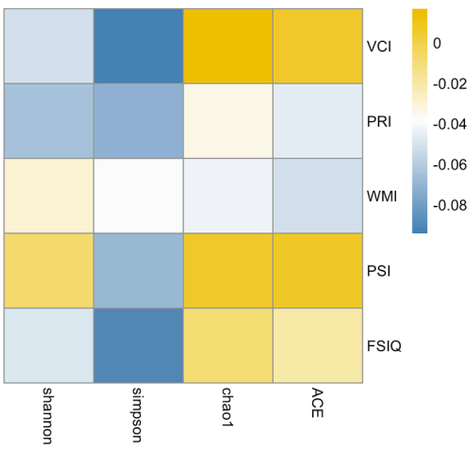

Figure S4 Multiple linear regression analysis of the association of alpha-diversity indexes with intelligence scores. Results were adjusted for age, sex, fiber intake and corrected for multiple testing. The β coefficient are indicated by the color of the cells. Positive or negative correlations are represented by shades of yellow or blue respectively. VCI, Verbal Comprehension Index; PRI, Perceptual Reasoning Index; WMI, Working Memory Index; PSI, Processing Speed Index; FSIQ, Full-Scale Intelligence Quotient; ACE, abundance-based coverage estimator.

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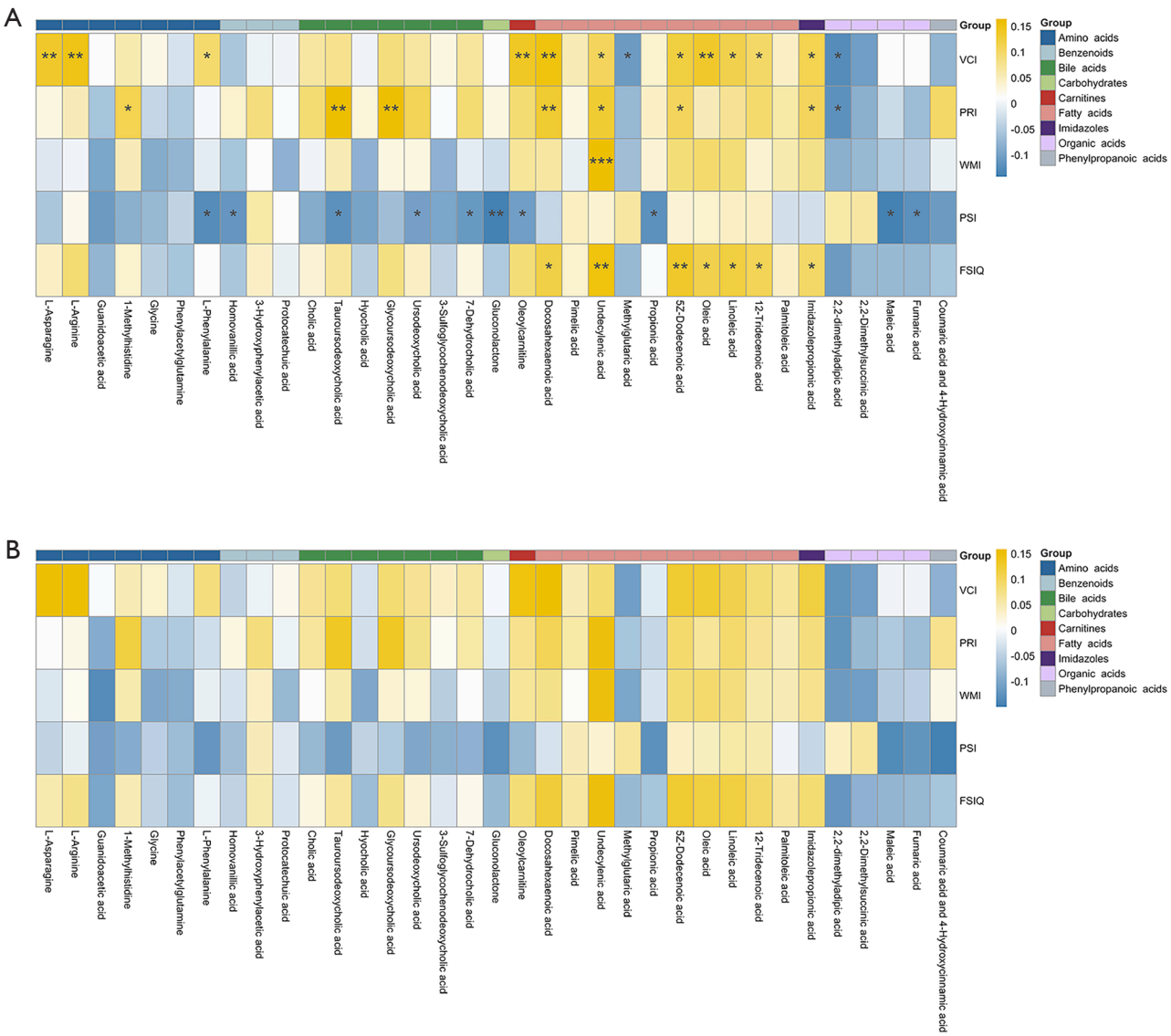

Figure S5 Spearman correlation and multiple linear regression analysis of the association of selected fecal metabolites with intelligence scores. (A) Spearman correlation analysis of the association of selected fecal metabolites with intelligence scores. (B) Multiple linear regression analysis of the associations of selected fecal metabolites with intelligence indexes after adjustment for age, sex and correction for multiple testing. The Spearman's rank correlation coefficient and β coefficient are indicated by the color of the cells. Positive or negative correlations and β coefficient are represented by shades of yellow or blue respectively. *, P<0.05; **, P<0.01; ***, P<0.001. VCI, Verbal Comprehension Index; PRI, Perceptual Reasoning Index; WMI, Working Memory Index; PSI, Processing Speed Index; FSIQ, Full-Scale Intelligence Quotient.

https://dx.doi.org/10.21037/tp-22-610