Amino Acids
DOI 10.1007/s00726-016-2308-y

ORIGINAL ARTICLE

Metabolic shifts and structural changes in the gut microbiota upon branched-chain amino acid supplementation in middle-aged mice

Zhan Yang[1] · Simo Huang[1] · Dayang Zou[1] · Derong Dong[1] · Xiaoming He[1] · Ningwei Liu.[1] · Wei Liu[1] · Liuyu Huang[1]

Received: 3 May 2016 / Accepted: 4 August 2016

Abstract The importance of gut microbiota to health has gained extensive attention and is strongly correlated with diet. Dietary supplementation with a branched-chain amino acid-enriched mixture (BCAAem) exerts a variety of beneficial effects in mice and humans. In mice, BCAAem supplementation can promote longevity, but its influence on the gut ecosystem and the underlying mechanism remain unclear. To address this issue, BALB/C mice were fed a BCAAem-supplemented diet and their gut microbiomes were analysed by 16S rDNA sequencing. Quantitative polymerase chain reaction was performed to identify *Bifidobacterium* spp. in the gut, and gas chromatography–mass spectrometry was conducted for faecal-metabolite detection. The results showed that the structure of the gut microbiota changed, and BCAAem-supplementation in mice slowed the change speed of gut microbiota which is due to age. In addition, the abundance of the *Akkermansia* and *Bifidobacterium* increased in BCAAem-supplemented mice, while the ratio of Enterobacteriaceae decreased in BCAAem-supplemented mice. Moreover, 12 different metabolites, representing sugar and lipid metabolism, were altered between the supplemented and control groups. Thus, BCAAem influences the gut microbiota and gut metabolism. In addition, the BCAAem-supplemented group presented lower serum concentrations of lipopolysaccharide-binding protein. The changes are indicative of lower antigen loads in the host gut. These results suggest that dietary supplementation with BCAAem may be considered for improving health and promoting healthy aging.

Keywords Branched-chain amino acid · Dietary supplement · Gut microbiota · Metabolism · Mice

Introduction

The gut microbiota, which plays an essential role in maintaining individual health, is present in the gastrointestinal tract, comprised of a vast variety of microorganisms present in huge amounts. It has been recognized as an integral part of the host, constituting a so-called "metaorganism" (Turnbaugh et al. 2007; Qin et al. 2010). However, the composition of the microbiota is not constant during the lifetime of host and changes with age for many reasons such as decreased intestinal motility (Kleessen et al. 1997), gut fermentative processes (Brocklehurst 1972; Macfarlane et al. 1989), and inflammaging (Franceschi 2007; Franceschi et al. 2007; Larbi et al. 2008; Ostan et al. 2008). The gut microbiota shows plasticity and is mostly influenced by an individual's diet (Sonnenburg et al. 2004; Zhang et al. 2010; van Hylckama Vlieg et al. 2011). Therefore, the gut microbiota may be a potentially useful target for strategies to improve the health of the aged population.

Supplementation with a branched-chain amino acid-enriched mixture (BCAAem) has been shown to promote health and extend the lifespan of mice (D'Antona et al. 2010). It may also provide health benefits similar to those conferred by calorie restriction (CR) (Valerio et al. 2011), a method that is recognized as the gold standard for lifespan extension in addition to other beneficial

Handling Editor: J. González López.

✉ Wei Liu
liuwei5269@qq.com

✉ Liuyu Huang
huangliuyuly@163.com

[1] Institute of Disease Control and Prevention, Academy of Military Medical Sciences, No. 20 Dongda Street, Fengtai District, Beijing, China

Published online: 18 August 2016

effects (Guarente 2008). Therefore, CR and BCAAem supplementation may have similar mechanisms, including increased mitochondrial respiration and reduced production of oxygen radicals (Nisoli et al. 2005; Civitarese et al. 2007; Ristow et al. 2009; D'Antona et al. 2010; Valerio et al. 2011). A recent study showed that CR could reshape the structure of the gut microbiota in mice (Zhang et al. 2013), indicating a potential association between CR, BCAAem supplementation, the gut ecosystem, and longevity. However, the mechanism by which BCAAem supplementation influences the gut has not yet been elucidated. In this study, we investigated the structural and metabolic changes in the gut microbiota of mice in response to BCAAem supplementation. Toward this objective, we applied 16S rDNA sequencing and quantitative polymerase chain reaction (qPCR) to analyse the gut microbiota, and gas chromatography-mass spectrometry (GC–MS) was used to analyse the faecal metabolome of the mice. The ultimate goal of this work was to provide a scientific basis for the use of BCAAem supplementation as an effective dietary intervention to improve the gut microbiota as well as the health of aging people.

Materials and methods

Animals and treatment

Eighteen male BALB/c mice (age 10 months, weight 32 ± 1.3 g) were obtained from Vital River Laboratories (Beijing, China). The mice were housed in a specific pathogen-free animal experiment facility at 23 ± 1 °C and 53 ± 2 % humidity with a 12-h light/dark cycle. The mice were fed a standard laboratory diet (ad libitum) in individual standard stainless steel cages. Previous studies have suggested that the gut microbiota, lifespan, and immune responses are also different between the sexes (Morell 1995; Barrett and Richardson 2011; Markle et al. 2013). To eliminate sex as an influencing factor in our experiments, we used only male BALB/c mice in this study. Therefore, it is important to bear in mind that the results for female mice may be different. The mice were randomly assigned to two groups: (1) unsupplemented (control, $n = 9$) and (2) supplemented with BCAAem (in drinking water; 1.5 mg/g body weight per day) beginning at 11 months of age ($n = 9$). The BCAAem supplement contained (in %): leucine 31.3; lysine 16.2; isoleucine 15.6; valine 15.6; threonine 8.8; cysteine 3.8; histidine 3.8; phenylalanine 2.5; methionine 1.3; tyrosine 0.7; and tryptophan 0.5. The contents of BCAAem supplement were described previously (Solerte et al. 2008a, b; D'Antona et al. 2010). The mice drank 5 ± 1 mL of water daily, and the BCAAem solution was prepared at a concentration of 0.01 g/mL.

Faecal sample collection and extraction

Fresh faecal samples were collected at the age of 11 (before BCAAem supplementation) and 15 months from both the supplemented and control groups, using a metabolic cage, and were stored at −80 °C. For 16S rDNA sequencing, total microbial DNA of the samples was extracted using QIAamp DNA Stool Mini Kit (Qiagen, Germany) following the manufacturer's protocol. The final DNA concentration was determined spectrophotometrically in an ND-1000 system (Thermo Fisher Scientific, USA).

For GC–MS analysis, stool samples were collected only from the 15-month-old mice from both the supplemented and control groups. The stool sample (200 µg) was suspended in 600 µL of methanol and then centrifuged at 10,000×g for 15 min to remove insoluble material. The supernatant was then passed through a 0.22-µm filter, transferred into a clean Eppendorf tube, and stored at −80 °C until GC–MS analysis.

Sequencing and bioinformatics

The 16S rRNA V3 and V4 amplicons were sequenced on the MiSeq 2.0 platform (Illumina, USA) according to the manufacturer's protocol. Sample reads were organized and evaluated using the CASAVA1.8 software. Chimeric sequences were identified and removed with FASTX Toolkit 0.0.13 (http://hannonlab.cshl.edu/fastx_toolkit/index.html). Operational taxonomic units (OTUs) were identified with a 97 % similarity threshold, using mothur software (http://www.mothur.org/). Beta diversity was determined according to the taxonomic classification at the genus level, and the gut bacteria were analysed at each level.

Quantitative polymerase chain reaction (qPCR)

qPCR was performed with SYBR Green (SYBR Green premix Ex Taq, Takara, Japan) in the CFX Connect Real-Time System (Bio-Rad, USA). The thermocycling conditions and specific primers for *Bifidobacterium* have been described previously (Kaufmann et al. 1997; Kullen et al. 2000; Marteau et al. 2001; Baker et al. 2003; Matsuki et al. 2004; Rinttilä et al. 2004; Collado et al. 2007). Primers

with the following sequences were used: Bifidobacterium-F CTCCTGGAAACGGGTGG; Bifidobacterium-R GGTGTTCTTCCCGATATCTACA. The final sample volume for qPCR was 25 μL, containing 200 ng of template DNA extracted from the faecal samples. Melting curve analysis was performed to confirm the specificity of amplification. The copy number of *Bifidobacterium* genes in the faecal samples was determined by comparing the threshold cycle values of samples to those of the standard curves. The standards were prepared by amplifying the gene from *Bifidobacterium* purifying the amplicons with the TIANGEN PCR purification kit (TIANGEN Biotech Co. Ltd., Beijing, China), and then cloning them in the PGM-T Vector, using the PGM-T Clone kit (TIANGEN Biotech Co. Ltd.). Plasmid DNA was extracted using the TIANprep Mini Plasmid Kit (TIANGEN Biotech Co. Ltd.), and the concentration was measured on a NanoDrop ND1000 spectrometer (Thermo Fisher Scientific). Plasmid DNAs with 10-fold dilution were used as standards. Statistical analysis of the qPCR data between groups was performed with log-transformed data, and the Student *t* test was performed to determine the statistical differences between the groups, in which $P < 0.05$ was considered significant.

GC–MS analysis of faecal extracts

The faecal extracts (100 μL) were vortexed thoroughly, and 10 μL of a ribitol stock solution (0.2 mg/mL H_2O) was added as an internal reference. The mixture was placed on a shaker at 70 °C for 15 min and centrifuged at 10,000×*g* for 10 min. The supernatant was mixed with 500 μL of pure water and 250 μL of chloroform, and then centrifuged again at 2500×*g* for 15 min. The upper (polar) phase was removed and evaporated to dryness under a stream of nitrogen in a thermostatically controlled water bath (60 °C). Metabolites in the faecal samples were derivatized prior to GC–MS analysis as described previously (Fiehn et al. 2000). Methoxyamine hydrochloride (20 μL of a solution containing 20 mg/mL pyridine) was then added to the dried fraction of the polar phase. Following continuous shaking at 30 °C for 90 min, 40 μL of *N*-methyl-*N*-trimethylsilyl trifluoroacetamide was added, and the tube was incubated at 37 °C for 30 min. All chemicals were from Sigma-Aldrich Chemical Co. (Steinheim, Germany).

After further incubation for 120 min at 23 °C, 0.3 μL of each sample was injected into a 30-m DB-5 column at a split ratio of 25:1. The GC–MS system consisted of an HP 6890 gas chromatograph and a time-of-flight mass spectrometer (Waters, USA). The injection temperature was 230 °C, the interface temperature was 290 °C, and the ion source temperature was 220 °C, with an electron energy of 70 eV. Helium, the carrier gas, was set to a column flow rate of 1 mL/min. After a 5-min solvent delay time at 70 °C, the oven temperature was increased to 310 °C at 5 °C/min, followed by a 1-min isocratic cool down to 70 °C and an additional 5-min delay. Mass Lynx software (Waters) was used to acquire the chromatographic data. NIST02 libraries with electron impact spectra were searched rigorously for all peaks detected with the total ion current to identify the metabolites. Compounds were also identified by comparison of their mass spectra and retention times with those of commercially available reference compounds.

Data processing and analyses

To effectively compare the data collected from the faecal samples, ribitol was added to each sample as an internal standard to correct for minor variations during sample preparation and analysis. The data were analysed with principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA), using MarkerLynx applications manager software (Waters), the most commonly used algorithm in metabolomics research. This software incorporates a peak deconvolution package that allows for the detection and retention-time alignment of the peaks eluted in each data file across the entire data set. MarkerLynx extracts components using mass chromatograms and lists the detected peaks according to their masses and retention times, together with their associated intensities. All data were exported to data matrix in a.txt file format. In the data matrix, the sample IDs were considered observations, the peaks and retention times were considered the response variables, and then the data were imported into the SIMCA-P + program (version 10.0, Umetrics, Sweden) to perform multivariate statistical analysis, including PCA and OPLS-DA. The simultaneous comparison of a large number of complex objects was facilitated by reduction of the dimensionality of the data set with two-dimensional projection procedures. Differences in metabolites were also analysed and compared between the two groups, using variable importance in projection (VIP, a value that indicates the relative contributions of metabolites to the grouping) to evaluate them.

Detection of lipopolysaccharide binding protein (LBP)

Blood samples were also collected from the tail veins of these mice after overnight fasting. The blood was

centrifuged at 10,000×*g* for 30 min and LBP levels in the serum were determined using the Mouse LBP ELISA Kit (Cell Sciences, USA) according to the manufacturer instructions. The Student t-test was performed to determine the statistical differences between the groups; $P < 0.05$ was considered significant.

Results

Body weights of mice in the BCAAem-supplement group and the control group

The mice were acclimated for 4 weeks and studied experimentally at 11 months of age. The mice were weighed at 11 and 15 months of age in both the BCAAem-supplement group and control group. At 11 months of age, the average body weights of mice in the supplement group and control group were 32.5 ± 1.7 g and 32.4 ± 1.8 g, respectively. At 15 months of age, the average body weights in the supplement group and control group were 32.8 ± 1.3 g and 32.5 ± 1.3 g, respectively. No significant differences were observed between these groups at either time point.

Sequencing results for the faecal samples

A total of 31 faecal samples (17 from 11-month-old mice and 14 from 15-month-old mice) were collected and sequenced. After OTU identification and chimera filtering, 2,554,003 reads were assigned to 171,365 OTUs, with each sample containing 5528 OTUs and 82,387 sequences on average.

Differences in gut bacterial communities upon BCAAem supplementation

The OTUs of all the samples were quantified and compared, and no significant difference in the number of OTUs was found between the BCAAem-supplemented and control groups at 11 months of age ($P = 0.23$). However, the BCAAem-supplemented group had significantly higher numbers of OTUs than did the control group ($P < 0.05$) at 15 months of age, indicating a richer microbial diversity in the supplemented group. And it also shows in both BCAAem-supplemented and control groups, the mice had higher numbers of OTUs at 11 months than that in 15 months ($P < 0.05$), indicating a lower microbial diversity due to age. The results are shown in Table 1.

The ratios of gut microbiota at the phylum level in the supplemented and control mouse groups (11- and 15-months-old) are shown in Table 2. Bacteroidetes and Firmicutes were the dominant phyla in both the supplemented and control groups, representing approximately 97.5 % of the total gut microbiota. In the control group, compared with proportions measured at 11 months, the average proportion of the Bacteroidetes phylum increased 8.27 %, while that of the Firmicutes phylum decreased 8.08 % at 15 months. In the supplemented group, the average proportion of the Bacteroidetes phylum increased 4.17 %, while that of the Firmicutes phylum decreased 3.13 % at 15 months. At 15 month of age, compared with the control group, the proportion of the Bacteroidetes phylum decreased 3.9 % in the supplemented group, while the Firmicutes phylum increased in proportion by 3.62 %. Changes in relative abundance of the two phyla in the control group occurred because of age. In the supplemented group, the rate of change in the two dominant phyla due to age was slowed by BCAAem supplementation. As the Bacteroidetes and Firmicutes are the dominant phyla in the gut, their relative ratios may be used as a structural indicator of the gut microbiota. In the control group, the Firmicutes/Bacteroidetes ratio at 11 months of age was 0.90, while that at 15 months was 0.64. In the supplemented group, the Firmicutes/Bacteroidetes ratio at 11 months was 0.87, while that at 15 months was 0.75. Thus, at 15 months of age, the Firmicutes/Bacteroidetes ratio of the supplemented group was higher than the control group and was closer to the ratio observed in 11-month-old mice. In addition, it is worth mentioning that as the third most abundant phylum, the proportion of Proteobacteria in the 15-month-old supplemented group was much lower than that measured in the control group. The Proteobacteria phylum includes some pathogenic bacteria such as enterobacteria, which are considered pathobionts and can cause pathogenic infections in certain circumstances (Pédron and Sansonetti 2008); thus, BCAAem supplementation may help control the presence of pathogenic bacteria in the gut microbiota.

The ratios of gut microbiota at the class level for the supplemented and control groups at 11 and 15 months of age are shown in Table 3. Bacteroidia, Clostridia, Bacilli were the dominant classes of gut microbiota. As the dominant classes of the Bacteroidetes and Firmicutes phyla, respectively, Bacteroidia and Clostridia showed the same

Table 1 OUT numbers for specific bacterial groups

	OUT numbers		*P* value
	BCAAem supplement group	Control group	
11 month	6256 ± 455.8	6078 ± 485.4	0.23
15 month	5056 ± 422.5	4435 ± 623.5	0.03
P value	4.76E−05	6.82E−05	

Table 2 Average percentages of gut microbiota at the phylum level in two groups at 11 and 15 months

Bacteria phylum	BCAAem-11 m	Control-11 m	BCAAem-15 m	Control-15 m
Other	0.003	0.004	0.004	0.004
Actinobacteria	0.081	0.056	0.025	0.048
Bacteroidetes	51.68	51.37	55.85	59.64
Cyanobacteria	0.226	0.275	0.164	0.094
Deferribacteres	0.588	0.189	0.267	0.134
Firmicutes	44.91	46.24	41.78	38.16
Fusobacteria	0.000	0.000	0.000	0.002
Proteobacteria	0.763	0.750	0.648	1.336
TM7	0.219	0.949	0.321	0.485
Tenericutes	1.481	0.147	0.684	0.091
Verrucomicrobia	0.054	0.023	0.268	0.009

Table 3 Average percentages of gut microbiota at the class level in two groups at 11 and 15 months

Bacteria class	BCAA-11 m	Control-11 m	BCAA-15 m	Control-15 m
Actinobacteria	0.01	0.00	0.00	0.00
Coriobacteriia	0.07	0.05	0.02	0.04
Bacteroidia	51.68	51.36	55.84	59.64
4C0d-2	0.19	0.24	0.15	0.08
Chloroplast	0.04	0.03	0.02	0.02
Deferribacteres	0.59	0.19	0.27	0.13
Bacilli	0.49	0.35	0.21	0.34
Clostridia	44.38	45.79	41.51	37.72
Erysipelotrichi	0.04	0.10	0.06	0.10
Alphaproteobacteria	0.05	0.02	0.02	0.01
Betaproteobacteria	0.04	0.08	0.01	0.03
Deltaproteobacteria	0.21	0.50	0.35	0.32
Epsilonproteobacteria	0.34	0.05	0.16	0.06
Gammaproteobacteria	0.13	0.10	0.11	0.92
TM7-3	0.22	0.95	0.32	0.49
Mollicutes	1.48	0.15	0.68	0.09
Verrucomicrobiae	0.05	0.02	0.27	0.01

trends in abundance as did the Bacteroidetes and Firmicutes phyla. Bacilli is a class of bacteria class in the Firmicutes phylum and includes some bacteria that are closely related to poor health such as streptococci, staphylococci, and enterococci. We showed that at 15 months of age, the supplemented group had a lower ratio of Bacilli than did the control group; thus, BCAAem supplementation may help reduce the prevalence of Bacilli in the gut.

The ratios of gut microbiota at the order level for the supplemented and control groups at 11 and 15 months of age are shown in Table 4. Bacteroidales, Clostridiales, Deferribacterales, Lactobacillales, Desulfovibrionales and CW040 were the dominant orders of the gut microbiota. Bacteroidales and Clostridiales showed the same variation trends as did Bacteroidia and Clostridia at 15 months of age, compared with control group (i.e., Bacteroidales decreased 3.794 % and Clostridiales increased 3.79 %). Interestingly, these dominant orders did not show the greatest change. In the control group at 15 month of age, the ratio of Enterobacteriales was 0.91 %, representing a >tenfold increase over the prevalence at 11 months of age, whereas this ratio was 0.10 % in the supplemented group. At 15 months of age, the prevalence of Enterobacteriales increased sharply due to old age in BALB/c mice, but in the supplemented group at 15 months, Enterobacteriales were present at only slightly higher levels than measured at 11 month of age.

Table 4 Average percentages of gut microbiota at the order level in two groups at 11 and 15 months

Bacteria order	BCAAem-11 m	Control-11 m	BCAAem-15 m	Control-15 m
Actinomycetales	0.01	0.00	0.00	0.01
Coriobacteriales	0.07	0.05	0.02	0.04
Bacteroidales	51.68	51.36	55.84	59.64
Flavobacteriales	0.00	0.01	0.00	0.00
YS2	0.19	0.24	0.15	0.08
Streptophyta	0.04	0.03	0.02	0.02
Deferribacterales	0.59	0.19	0.27	0.13
Lactobacillales	0.48	0.34	0.20	0.34
Clostridiales	44.38	45.79	41.51	37.72
Erysipelotrichales	0.04	0.10	0.06	0.10
RF32	0.04	0.02	0.02	0.01
Burkholderiales	0.04	0.07	0.01	0.02
Neisseriales	0.00	0.00	0.00	0.01
Desulfovibrionales	0.21	0.50	0.35	0.32
Campylobacterales	0.34	0.05	0.16	0.06
Enterobacteriales	0.05	0.08	0.10	0.91
Pasteurellales	0.01	0.01	0.01	0.01
Pseudomonadales	0.05	0.01	0.01	0.00
Xanthomonadales	0.02	0.00	0.00	0.00
CW040	0.22	0.95	0.32	0.49
Anaeroplasmatales	1.43	0.13	0.67	0.08
RF39	0.05	0.02	0.2	0.01
Verrucomicrobiales	0.05	0.02	0.27	0.01

The ratios of the gut microbiota at the family level for the supplemented and control groups at 11 and 15 months of age are shown in Table 5. An unknown family of Clostridiales order, Rikenellaceae, S24-7, Bacteroidaceae, Lachnospiraceae, and Ruminococcaceae were the dominant families of the gut microbiota. At 15 months of age, in the supplemented group, the prevalence of Prevotellaceae increased 1.929 % and that of Rikenellaceae increased 5.733 %, compared with the control group. The abundance-variation trend of Enterobacteriaceae was the same as observed with Enterobacteriales.

The ratios of the gut microbiota at the genus level for the supplemented and control groups at 11 and 15 months of age are shown in Table 6. An unknown genus of Clostridiales order, an unknown genus of S24-7 family, an unknown genus of Rikenellaceae family, Prevotella, and Bacteroides were the dominant genus of the gut microbiota. At 15 months of age, in the supplemented group, the prevalence of Prevotella increased 2.03 % and that of Bacteroides increased 1.27 %, compared with control group. At 15 months of age, in the supplemented group, the proportion of the Akkermansia genus was 0.27 %, which was markedly higher than the proportion of 0.01 % measured in the control group. Members of the Akkermansia genus can potentially reverse high-fat diet-induced metabolic disorders and insulin resistance, and also increase the intestinal levels of endocannabinoids that control inflammation, the gut barrier, and gut peptide secretion (Everard et al. 2013). Thus, BCAAem supplementation can increase the ratio of Akkermansia in the gut.

qPCR results for bifidobacteria in the gut

Bifidobacteria are commonly recognized as health-promoting bacteria; however, our sequencing results indicated that the abundance of Bifidobacterium was very low (<0.001 %), making its prevalence difficult to compare in the two groups. For this reason, qPCR was performed with faecal samples to specifically detect the presence of *Bifidobacterium*. The abundance of bifidobacteria in 15-month-old mice was lower than in 11-month-old mice, but in the supplemented group, bifidobacteria were more abundant than in the control group ($P < 0.05$). The average numbers of DNA copies in the two groups are shown in Fig. 1.

Table 5 Average percentages of gut microbiota at the family level in two groups at 11 and 15 months

Bacteria family	BCAAem-11 m	Control-11 m	BCAAem-15 m	Control-15 m
Corynebacteriaceae	0.01	0.00	0.00	0.00
Coriobacteriaceae	0.07	0.05	0.02	0.04
o__Bacteroidales;Other	0.34	0.21	0.26	0.27
o__Bacteroidales;f__unknown	3.71	2.19	2.88	4.08
Bacteroidaceae	5.10	5.24	4.58	5.84
Porphyromonadaceae	0.13	0.10	0.07	0.04
Prevotellaceae	7.40	7.92	10.90	9.00
Rikenellaceae	15.85	3.64	10.13	4.40
S24-7	15.42	30.50	24.28	33.93
Odoribacteraceae	3.05	0.80	1.93	0.87
Paraprevotellaceae	0.68	0.76	0.83	1.23
Weeksellaceae	0.00	0.01	0.00	0.00
o__YS2;f__unknown	0.19	0.24	0.15	0.08
o__Streptophyta;f__unknown	0.04	0.03	0.02	0.02
Deferribacteraceae	0.60	0.19	0.27	0.13
Lactobacillaceae	0.44	0.32	0.20	0.30
Streptococcaceae	0.02	0.01	0.00	0.03
o__Clostridiales;Other	0.31	0.41	0.40	0.35
o__Clostridiales;f__unknown	32.90	31.32	29.80	25.58
Clostridiaceae	0.02	0.03	0.01	0.03
Dehalobacteriaceae	0.15	0.26	0.19	0.17
Lachnospiraceae	5.04	6.05	4.32	4.44
Peptococcaceae	0.03	0.06	0.04	0.04
Ruminococcaceae	5.92	7.62	6.74	7.09
Veillonellaceae	0.00	0.02	0.00	0.01
Mogibacteriaceae	0.02	0.03	0.02	0.02
Erysipelotrichaceae	0.04	0.10	0.06	0.10
o__RF32;f__unknown	0.04	0.02	0.02	0.10
Alcaligenaceae	0.00	0.07	0.01	0.02
Comamonadaceae	0.03	0.00	0.00	0.00
Neisseriaceae	0.00	0.00	0.00	0.01
Desulfovibrionaceae	0.21	0.50	0.35	0.32
Helicobacteraceae	0.34	0.05	0.16	0.06
Enterobacteriaceae	0.05	0.08	0.10	0.91
Pasteurellaceae	0.01	0.01	0.01	0.01
Moraxellaceae	0.04	0.01	0.00	0.00
Xanthomonadaceae	0.02	0.00	0.00	0.00
F16	0.22	0.95	0.32	0.49
Anaeroplasmataceae	1.43	0.13	0.67	0.08
o__RF39;f__unknown	0.05	0.02	0.02	0.01
Verrucomicrobiaceae	0.05	0.02	0.27	0.01

GC–MS analyses of the faecal samples

GC–MS chromatograms for the faecal samples from the supplemented and control groups were prepared, and 56 metabolites were identified, including sugars, amino acids, fatty acids, and organic acids. These metabolites have been implicated in multiple biochemical processes, including energy and substance metabolism. Thus, the

Table 6 Average percentages of gut microbiota at the genus level in two groups at 11 and 15 months

Bacteria genus	BCAAem-11 m	Control-11 m	BCAAem-15 m	Control-15 m
Corynebacterium	0.01	0.00	0.00	0.00
f__Coriobacteriaceae;g__	0.00	0.01	0.00	0.00
Adlercreutzia	0.07	0.05	0.02	0.04
o__Bacteroidales;Other;Other	0.34	0.21	0.26	0.27
o__Bacteroidales;f__;g__unknown	3.71	2.19	2.88	4.08
Bacteroides	5.10	5.24	4.58	5.84
Parabacteroides	0.13	0.10	0.07	0.04
f__Prevotellaceae;g__unknown	0.10	0.12	0.12	0.22
Prevotella	7.30	7.80	10.78	8.75
f__Rikenellaceae;Other	0.01	0.00	0.01	0.01
f__Rikenellaceae;g__unknown	15.52	3.56	9.92	4.11
AF12	0.04	0.00	0.02	0.01
Rikenella	0.28	0.07	0.18	0.27
f__S24-7;g__unknown	15.42	30.50	24.28	33.93
Odoribacter	3.05	0.80	1.93	0.87
Paraprevotella	0.05	0.06	0.06	0.04
Prevotella	0.62	0.70	0.78	1.19
Chryseobacterium	0.00	0.01	0.00	0.00
o__YS2;f__;g__unknown	0.19	0.24	0.15	0.08
o__Streptophyta;f__;g__unknown	0.04	0.03	0.02	0.02
Mucispirillum	0.59	0.19	0.27	0.13
Carnobacterium	0.01	0.01	0.00	0.00
Lactobacillus	0.44	0.32	0.20	0.30
Lactococcus	0.01	0.01	0.00	0.0
Streptococcus	0.01	0.00	0.00	0.00
o__Clostridiales;Other;Other	0.31	0.41	0.39	0.35
o__Clostridiales;f__;g__unknown	32.90	31.32	29.80	25.58
f__Clostridiaceae;g__unknown	0.01	0.01	0.00	0.01
Clostridium	0.01	0.02	0.01	0.02
Dehalobacterium	0.14	0.26	0.19	0.17
f__Lachnospiraceae;Other	0.28	0.27	0.23	0.305
f__Lachnospiraceae;g__ unknown	3.92	4.77	3.39	3.23
Blautia	0.01	0.01	0.01	0.01
Butyrivibrio	0.00	0.01	0.00	0.00
Coprococcus	0.38	0.43	0.28	0.39
Dorea	0.04	0.08	0.03	0.06
Roseburia	0.01	0.04	0.01	0.01
Ruminococcus	0.41	0.43	0.36	0.43
f__Peptococcaceae;g__ unknown	0.03	0.06	0.04	0.04
f__Ruminococcaceae;Other	0.24	0.32	0.22	0.31
f__Ruminococcaceae;g__ unknown	2.51	2.89	2.81	3.14
Oscillospira	2.47	3.61	2.89	2.96
Ruminococcus	0.69	0.79	0.81	0.67
Veillonella	0.00	0.01	0.00	0.01
f__Mogibacteriaceae;g__ unknown	0.01	0.03	0.02	0.02
f__Erysipelotrichaceae;g__ unknown	0.04	0.10	0.06	0.10
Coprobacillus	0.00	0.00	0.00	0.01
o__RF32;f__;g__ unknown	0.04	0.02	0.02	0.01
Sutterella	0.00	0.07	0.01	0.02

Table 6 continued

Bacteria genus	BCAAem-11 m	Control-11 m	BCAAem-15 m	Control-15 m
f__Comamonadaceae;Other	0.01	0.00	0.00	0.00
Delftia	0.02	0.00	0.00	0.00
Neisseria	0.00	0.00	0.00	0.01
f__Desulfovibrionaceae;Other	0.04	0.08	0.07	0.06
f__Desulfovibrionaceae;g__ unknown	0.02	0.03	0.03	0.02
Bilophila	0.02	0.05	0.08	0.04
Desulfovibrio	0.14	0.35	0.16	0.20
f__Helicobacteraceae;Other	0.01	0.01	0.01	0.00
f__Helicobacteraceae;g__ unknown	0.02	0.00	0.00	0.00
Flexispira	0.15	0.03	0.12	0.03
Helicobacter	0.16	0.01	0.03	0.02
f__Enterobacteriaceae;Other	0.01	0.01	0.01	0.06
f__Enterobacteriaceae;g__ unknown	0.03	0.07	0.08	0.78
Klebsiella	0.00	0.00	0.00	0.06
Proteus	0.01	0.00	0.00	0.00
Aggregatibacter	0.01	0.01	0.01	0.01
Acinetobacter	0.04	0.01	0.00	0.00
f__F16;g__ unknown	0.22	0.95	0.32	0.49
Anaeroplasma	1.43	0.13	0.67	0.08
o__RF39;f__;g__ unknown	0.05	0.02	0.02	0.01
Akkermansia	0.05	0.02	0.27	0.01

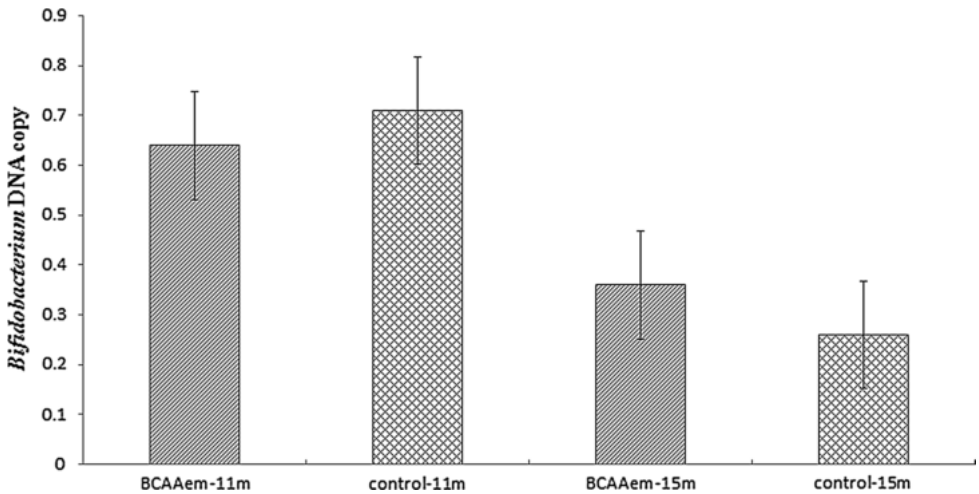
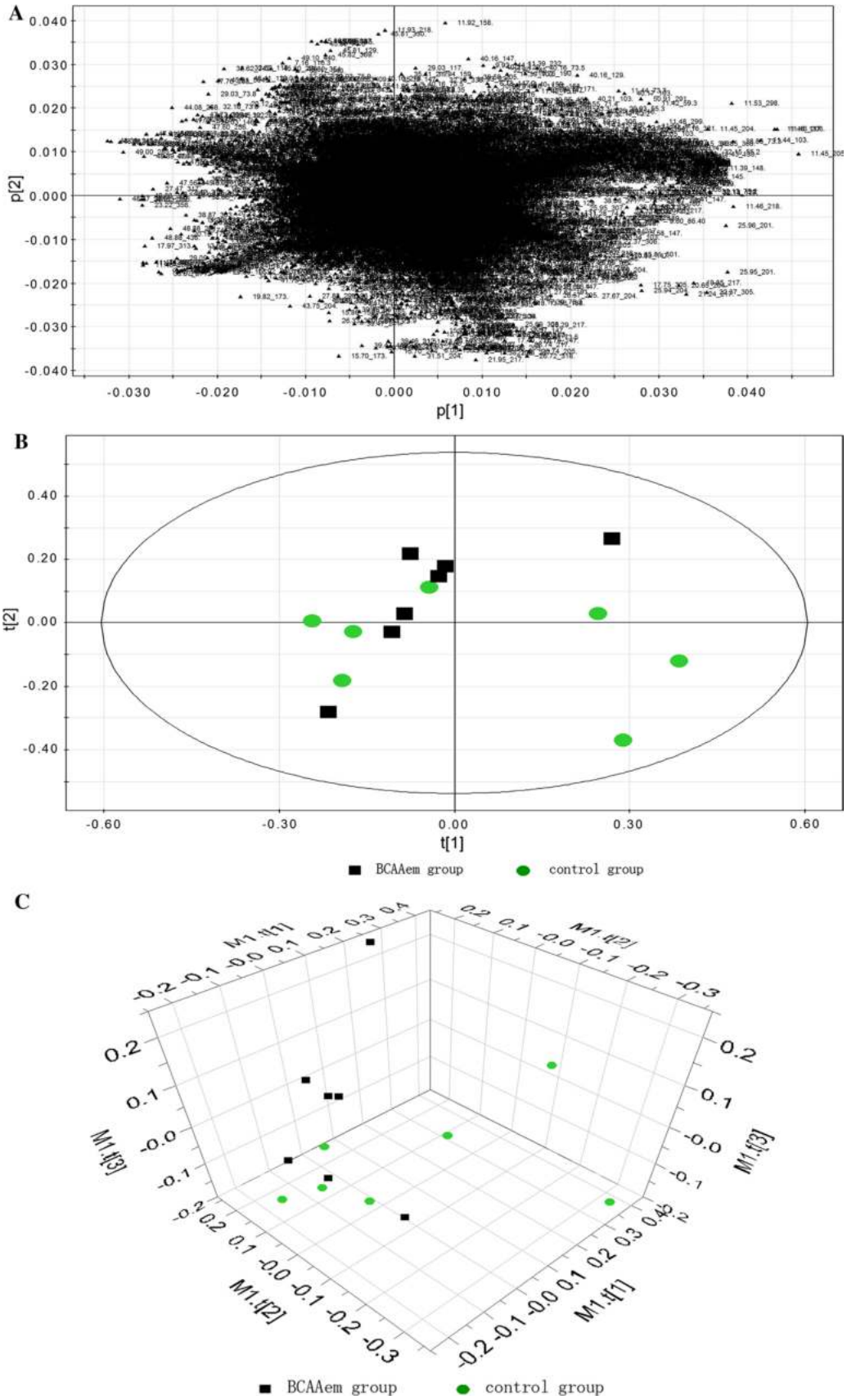


Fig. 1 The DNA copy numbers of *Bifidobacterium* species in the two groups at 11 and 15 months. At 11 months, in the supplemented group, the copy number was 0.64, in the control group, the copy number was 0.71, the *P* value was 0.26. At 15 months, in the supplemented group and control group, the copy numbers were 0.36 and 0.26, respectively, the *P* value was 0.04

chromatograms are considered to be chemical fingerprints describing the metabolic changes induced by BCAAem supplementation. Owing to the complexity of faecal metabolites, the differences in the chromatograms between the supplemented and control groups were relatively small in this study.


A
p[2]
p[1]
B
t[2]
t[1]
BCAAem group
control group
C
M1.t[1]
M1.t[2]
M1.t[3]
BCAAem group
control group

◂**Fig. 2** Loadings *plot* and the corresponding scores *plot* drawn using SIMCA-P + software for principal components analysis of gas chromatography/time-of-flight mass spectrometry data, derived for all BALB/c mice. **a** Loadings scatter *plot* for the branched-chain amino acid-enriched mixture (BCAAem)-supplemented and control groups. **b** Scores scatter *plot* for the BCAAem-supplemented and control groups. **c** Three-dimensional version of the scores *scatter plot*

PCA and OPLS-DA

PCA is a multivariate statistical method by which a data set with hundreds of variables can be represented by a few new variables (called principal components). These new variables are linear combinations of the original variables, without much loss of information contained in the original data set. All of the GC–MS data for the faecal and plasma samples of the control and supplemented groups were analysed in this manner. Loading scatter plot (Fig. 2a) and score scatter plot (Fig. 2b) were used to represent the sample distribution and clustering. The loading scatter plot represents the impact of each metabolite on the clustering and the score scatter plot represents the clustering in the new multivariate space, the three-dimensional version is shown in Fig. 2c.

The OPLS-DA score plots revealed clearer discrimination among the groups by removing the noise and uncorrelated variation compared to the PCA score plots (Fig. 3a–c). Analysis with the SIMCA-P + software indicated that there were marked changes in octadecenoic acid, cholesterol, oleic acid, urea, deoxycholic acid, glycerol, fructose, sucrose, erythritol, urea, 1-monooleoylglycerol, xylitol, phenyllactic acid, and stigmasterol resulting from supplementation with BCAAem. Fructose, sucrose, and oleic acids showed the highest VIP values (Table 7), indicating that these metabolites had the greatest impact on each principal component in the experimental group. Determining VIP values can identify significant metabolites in complex data sets from metabolomics data. When comparing differences between groups, higher VIP values correlate with greater significance. Thus, analytes selected by PCA can be confirmed by determining VIP values as being truly significant (Trivedi and Iles 2012).

Concentrations of serum LBP

The concentrations of LBP measured in the serum are shown in Fig. 4. LBP is a soluble acute-phase protein that binds to lipopolysaccharide (LPS), and serum LBP levels can reflect the host inflammatory state (higher LBP levels correlate with higher antigen loads in the gut of the host). The contents of LBP were significantly lower in samples from the BCAAem-supplemented mice compared to those of controls ($P < 0.05$).

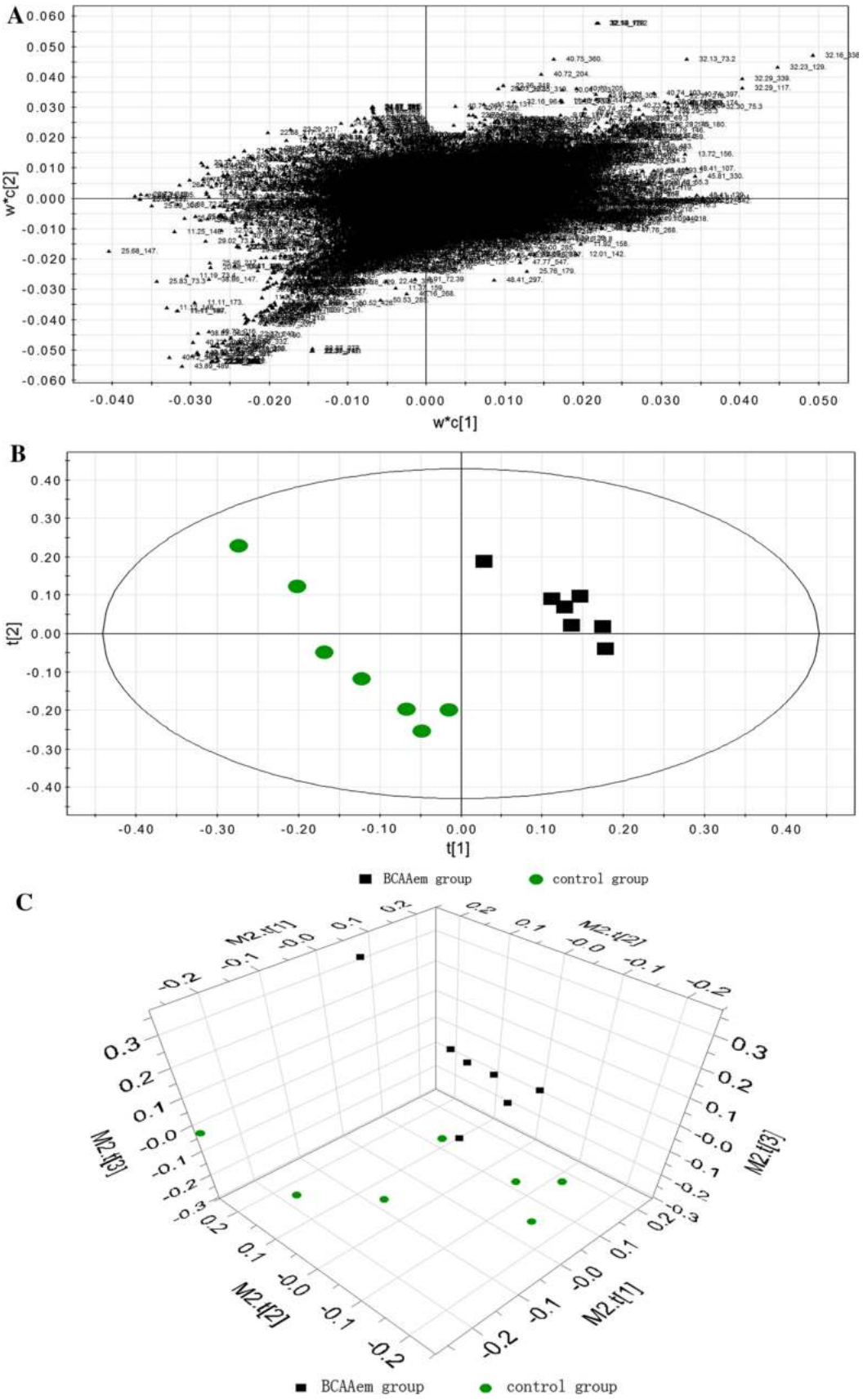
Discussion and conclusion

In this study, we investigated the structural and metabolic changes in the gut microbiota of mice brought about by BCAAem supplementation. BCAAem supplementation is known to extend the lifespan of mice (D'Antona et al. 2010), and has various health effects such as increasing fat leptin secretion and insulin sensitivity (Lynch et al. 2006; Zhang et al. 2007), decreasing body weight (Cota et al. 2006), and supporting mitochondrial biogenesis in the cardiac and skeletal muscles (D'Antona et al. 2010). To our knowledge, this is the first study to address whether the health effects of BCAAem supplementation are associated with changes to the gut microbiota.

Several studies have shown that the dominant phyla of human gut microbiota are *Firmicutes* and *Bacteroidetes*. Young adults have more *Firmicutes* than *Bacteroidetes*, but in aged people, *Bacteroidetes* is more abundant and the ratio of *Firmicutes*/*Bacteroidetes* is reduced (Mueller et al. 2006; Mariat et al. 2009; Zwielehner et al. 2009; Claesson et al. 2010; Mäkivuokko et al. 2010). Unlike in humans, the young BCAAem-supplemented BALB/c mice in this study had more *Bacteroidetes* than *Firmicutes*; however, the *Firmicutes*/*Bacteroidetes* ratio was similar to that measured in humans. Nevertheless, this ratio was higher than that in the control group, showing that the rate of change in the gut microbiota with age was slower in the BCAAem-supplemented group. The health effects of BCAAem mediated through the gut microbiota may occur through the increased prevalence of members of the Akkermansia and Bifidobacterium, as well as the inhibition of the increase of Enterobacteriaceae.

We also found that the faecal metabolites of the BCAAem-supplemented mice were significantly different from those in the control mice. The metabolites in the supplemented group were primarily sugars and lipids, indicating that BCAAem supplementation influences lipid and glycol metabolism in the gut microbiota, as well as in the body as a whole. As the metabolites changed in response to BCAAem supplementation, fructose, sucrose, and oleic acids had the highest VIP values, showing that the metabolic pathways associated with these compounds were the most significantly altered between the supplemented and control groups. Overall, the altered metabolites reflect the influence of BCAAem supplementation on gut metabolism and may serve as a useful guide toward elucidating the mechanism by which BCAAem influences the gut ecosystem.

Further, the BCAAem-supplemented group had lower concentration of LBP in the sera compared to the control


A
w*c[2]
w*c[1]
B
t[2]
t[1]
BCAAem group
control group
C
M2.t[1]
M2.t[2]
M2.t[3]
BCAAem group
control group

◂**Fig. 3** Loadings *plot* and the corresponding scores scatter *plot* drawn using SIMCA-P + software for orthogonal partial least-squares discriminant analysis (OPLS-DA) of gas chromatography/time-of-flight mass spectrometry data derived for all BALB/c mice. **a** Loadings scatter *plot* for the branched-chain amino acid-enriched mixture (BCAAem-)supplemented group and control groups. **b** Scores scatter *plot* for the BCAAem-supplemented and control groups. **c** Three-dimensional version of the scores scatter *plot*. The loadings *plot* and the corresponding scores scatter *plot* for OPLS-DA show obvious changes between the supplemented and control groups

Table 7 Changes in metabolites in faecal supernatants between the branched-chain amino acid-enriched mixture-supplemented and control groups analysed using SIMCA-P + software

Metabolite	Retention time (min)	VIP	*P* value
Octadecenoic acid	32.269	1.60836	0.000114
Cholesterol	45.854	1.3849	0.000306
Oleic acid	30.051	4.28684	0.001923
Deoxycholic acid	47.61	2.03722	0.005518
Glycerol	11.384	2.00242	0.007073
Fructose	25.668	7.69308	0.007208
Sucrose	38.869	4.39743	0.008826
Erythritol	17.383	1.15273	0.010927
1-monooleoylglycerol	40.736	1.29555	0.033833
Xylitol	22.383	1.11302	0.035445
Phenyllactic acid	20.585	1.86289	0.039064
Stigmasterol	47.47	2.78247	0.039201

VIP variable importance in projection

group. LBP is a soluble acute-phase protein that binds to LPS, the most abundant gut antigen with the most potent inflammation-provoking capacity. LBP elicits immune responses by presenting LPS to surface pattern-recognition receptors such as CD14 and Toll-like receptor 4 on immune cells (Sun et al. 2010). Thus, the LBP levels in serum may be a useful biomarker for the host inflammatory state (Zweigner et al. 2006). The lower concentrations of LBP in mice supplemented with BCAAem in this study indicate that BCAAem reduces the antigen load of the gut and, therefore, the host. LPS localizes to the outer membrane of gram-negative bacteria. We found that the Bacteroidetes and Proteobacteria phyla were both reduced in the BCAAem supplement group, as were the Betaproteobacteria and Gammaproteobacteria (including Enterobacteriaceae) classes, which belong to Proteobacteria phylum. These are all gram-negative bacteria, so the reduced LBP concentration in the supplemented group may have some relevance with the reduction of gram-negative bacteria levels. Interestingly, Ren et al. recently found that BCAA supplementation can increase the expression of endogenous intestinal β-defensin, which can regulate porcine LPS (Ren et al. 2016); thus, various approaches using BCAA have been tested to reduce LPS and LBP levels.

Overall, our data show that BCAAem supplementation influences the gut microbiota and gut metabolism, as well as the antigen load of the gut and host. Therefore, the health effects of BCAAem supplementation may be associated with its influence on the gut. Future studies should investigate further details of the influence of BCAAem supplementation on the gut, and include abundant sample quantities and rigorous experimental designs. The current results suggest that dietary supplementation with BCAAem may be considered for improving host health and promoting healthy aging.

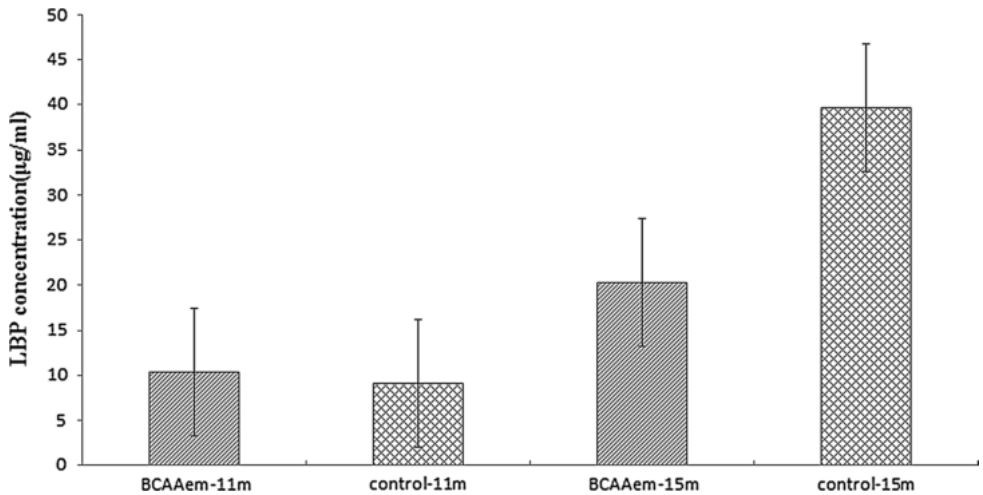


Fig. 4 Serum LBP concentrations in the two groups at 11 and 15 months. At 11 months, in the supplemented group, the LBP concentration was 10.3 μg/ml, in the control group, the LBP concentration was 9.1 μg/ml, the *P* value was 0.06. At 15 months, in the supplemented group and control group, the LBP concentrations were 20.3 and 39.7 μg/ml, respectively, the *P* value was 2.68E−14

Acknowledgments This work was supported by Mega-projects of Science and Technology Research of China (Grant No. 2013ZX10004-203) and the National Natural Science Foundation of China (Grant No. 81501836).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals The mouse experiments were conducted in accordance with the recommendations of the ethics provision for experiments on mice of the ethics committee of the Academy of Military Medical Sciences. The protocol was approved by the ethics committee of the Academy of Military Medical Sciences.

References

Baker GC, Smith JJ, Cowan DA (2003) Review and re-analysis of domain-specific 16S primers. J Microbiol Methods 55:541–555

Barrett EL, Richardson DS (2011) Sex differences in telomeres and lifespan. Aging Cell 10:913–921

Brocklehurst JC (1972) Bowel management in the neurologically disabled. The problem of old age. Proc R Soc Med 65:66–69

Civitarese AE, Carling S, Heilbronn LK et al (2007) Calorie restriction increases muscle mitochondrial biogenesis in healthy humans. PLoS Med 4:e76

Claesson MJ, Cusack S, O'Sullivan O et al (2010) Composition, variability, and temporal stability of the intestinal microbiota of the elderly. Proc Natl Acad Sci USA 108:4586–4591

Collado MC, Derrien M, Isolauri E, de Vos WM, Salminen S (2007) Intestinal integrity and Akkermansia muciniphila, a mucin-degrading member of the intestinal microbiota present in infants, adults, and the elderly. Appl Environ Microbiol 73:7767–7770

Cota D, Proulx K, Smith KA et al (2006) Hypothalamic mTOR signaling regulates food intake. Science 312:927–930

D'Antona G, Ragni M, Cardile A et al (2010) Branched-chain amino acid supplementation promotes survival and supports cardiac and skeletal muscle mitochondrial biogenesis in middle-aged mice. Cell Metab 12:362–372

Everard A, Belzer C, Geurts L et al (2013) Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity. Proc Natl Acad Sci USA 110:9066–9071

Fiehn O, Kopka J, Dörmann P, Altmann T, Trethewey RN, Willmitzer L (2000) Metabolite profiling for plant functional genomics. Nat Biotechnol 18:1157–1161

Franceschi C (2007) Inflammaging as a major characteristic of old people: can it be prevented or cured? Nutr Rev 65:S173–S176

Franceschi C, Giunta S, Olivieri F et al (2007) Inflammaging and anti-inflammaging: systemic perspective on aging and longevity emerged from studies in humans. Mech Ageing Dev 128:92–105

Guarente L (2008) Mitochondria–a nexus for aging, calorie restriction, and sirtuins? Cell 132:171–176

Kaufmann P, Pfefferkorn A, Teuber M, Meile L (1997) Identification and quantification of Bifidobacterium species isolated from food with genus-specific 16S rRNA-targeted probes by colony hybridization and PCR. Appl Environ Microbiol 63:1268–1273

Kleessen B, Sycura B, Zunft HJ, Blaut M (1997) Effects of inulin and lactose on fecal microflora, microbial activity, and bowel habit in elderly constipated persons. Am J Clin Nutr 65:1397–1402

Kullen MJ, Sanozky-Dawes RB, Crowell DC, Klaenhammer TR (2000) Use of the DNA sequence of variable regions of the 16S rRNA gene for rapid and accurate identification of bacteria in the *Lactobacillus acidophilus* complex. J Appl Microbiol 89:511–516

Larbi A, Franceschi C, Mazzatti D, Solana R, Wikby A, Pawelec G (2008) Aging of the immune system as a prognostic factor for human longevity. Physiology 23:64–74

Lynch CJ, Gern B, Lloyd C, Hutson SM, Eicher R, Vary TC (2006) Leucine in food mediates some of the postprandial rise in plasma leptin concentrations. Am J Physiol Endocrinol Metab 291:E621–E630

Macfarlane GT, Cummings JH, Macfarlane S, Gibson GR (1989) Influence of retention time on degradation of pancreatic enzymes by human colonic bacteria grown in a 3-stage continuous culture system. J Appl Microbiol 67:521–527

Mäkivuokko H, Tiihonen K, Tynkkynen S, Paulin L, Rautonen N (2010) The effect of age and non-steroidal anti-inflammatory drugs on human intestinal microbiota composition. Br J Nutr 103:227–234

Mariat D, Firmesse O, Levenez F et al (2009) The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. BMC Microbiol 9:123

Markle JG, Frank DN, Mortin-Toth S et al (2013) Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. Science 339:1084–1088

Marteau P, Pochart P, Dore J, Bera-Maillet C, Bernalier A, Corthier G (2001) Comparative study of bacterial groups within the human cecal and fecal microbiota. Appl Environ Microbiol 67:4939–4942

Matsuki T, Watanabe K, Fujimoto J, Takada T, Tanaka R (2004) Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in human feces. Appl Environ Microbiol 70:7220–7228

Morell V (1995) Zeroing in on how hormones affect the immune system. Science 269:773–775

Mueller S, Saunier K, Hanisch C et al (2006) Differences in fecal microbiota in different European study populations in relation to age, gender, and country: a cross-sectional study. Appl Environ Microbiol 72:1027–1033

Nisoli E, Tonello C, Cardile A et al (2005) Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS. Science 310:314–317

Ostan R, Bucci L, Capri M et al (2008) Immunosenescence and immunogenetics of human longevity. NeuroImmunoModulation 15:224–240

Pédron T, Sansonetti P (2008) Commensals, bacterial pathogens and intestinal inflammation: an intriguing ménage àtrois. Cell Host Microbes 3:344–347

Qin J, Li R, Raes J et al (2010) A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464:59–65

Ren M, Zhang S, Liu X et al (2016) Different lipopolysaccharide branched-chain amino acids modulate porcine intestinal endogenous β-Defensin expression through the Sirt1/ERK/90RSK pathway. J Agric Food Chem 64:3371–3379

Rinttilä T, Kassinen A, Malinen E, Krogius L, Palva A (2004) Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. J Appl Microbiol 97:1166–1177

Ristow M, Zarse K, Oberbach A et al (2009) Antioxidants prevent health-promoting effects of physical exercise in humans. Proc Natl Acad Sci USA 106:8665–8670

Solerte SB, Fioravanti M, Locatelli E et al (2008a) Improvement of blood glucose control and insulin sensitivity during a long term (60 weeks) randomized study with amino acid dietary supplements in elderly subjects with type 2 diabetes mellitus. Am J Cardiol 101(11A):82E–88E

Solerte SB, Gazzaruso C, Bonacasa R et al (2008b) Nutritional supplements with oral amino acid mixtures increases whole-body

lean mass and insulin sensitivity in elderly subjects with sarcopenia. Am J Cardiol 101(11A):69E–77E

Sonnenburg JL, Angenent LT, Gordon JI (2004) Getting a grip on things: how do communities of bacterial symbionts become established in our intestine? Nat Immunol 5:569–573

Sun L, Yu Z, Ye X et al (2010) A marker of endotoxemia is associated with obesity and related metabolic disorders in apparently healthy Chinese. Diabetes Care 33:1925–1932

Trivedi DK, Iles RK (2012) The Application of SIMCA P + in Shotgun Metabolomics Analysis of ZIC ®HILIC-MS Spectra of Human Urine Experience with the Shimadzu IT-TOF and Profiling Solutions Data Extraction Software. Chromat Separation Techniq 3:6

Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI (2007) The human microbiome project. Nature 449:804–810

Valerio A, D'Antona G, Nisoli E (2011) Branched-chain amino acids, mitochondrial biogenesis, and healthspan: an evolutionary perspective. Aging 3:464–478

van Hylckama Vlieg JE, Veiga P, Zhang C, Derrien M, Zhao L (2011) Impact of microbial transformation of food on health-from fermented foods to fermentation in the gastro-intestinal tract. Curr Opin Biotechnol 22:211–219

Zhang Y, Guo K, LeBlanc RE, Loh D, Schwartz GJ, Yu YH (2007) Increasing dietary leucine intake reduces diet-induced obesity and improves glucose and cholesterol metabolism in mice via multimechanisms. Diabetes 56:1647–1654

Zhang C, Zhang M, Wang S et al (2010) Interactions between gut microbiota, host genetics and diet relevant to development of metabolic syndromes in mice. ISME J 4:232–241

Zhang C, Li S, Yang L et al (2013) Structural modulation of gut microbiota in life-long calorie-restricted mice. Nat Commun 4:2163

Zweigner J, Schumann RR, Weber JR (2006) The role of lipopolysaccharide-binding protein in modulating the innate immune response. Microbes Infect 8:946–952

Zwielehner J, Liszt K, Handschur M, Lassl C, Lapin A, Haslberger AG (2009) Combined PCR-DGGE fingerprinting and quantitative-PCR indicates shifts in fecal population sizes and diversity of Bacteroides, bifidobacteria and Clostridium cluster IV in institutionalized elderly. Exp Gerontol 44:440–446