Available online at www.sciencedirect.com
ScienceDirect
Chinese Journal of Natural Medicines 2020, **18**(2): 90–102
doi: 10.1016/S1875-5364(20)30010-8
Chinese Journal of Natural Medicines

Profiling the mid-adult cecal microbiota associated with host healthy by using herbal formula *Kang Shuai Lao Pian* treated mid-adult mice

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Available online 20 Feb., 2020

[ABSTRACT] With the occurrence of aging process, decreased neuron dopamine, disrupted brown adipose tissue (BAT) remodeling and decreased butyrate level all reflect a weak host healthy in certain degree. Nevertheless, the signs of mid-adult gut microbiota, and its association with host healthy are not well understood. In current study, we deemed to illustrate the associations of age, neuron dopamine, BAT remodeling, butyrate and gut microbiota with the aid of traditional herbal formula *Kang Shuai Lao Pian* (KSLP), which is known for its anti-aging effect. Here, ELISA was performed to detect the production of brain dopamine, the mass of inguinal white adipose tissue versus interscapular brown adipose tissue (iWAT/iBAT) was calculated and considered as a sign of BAT remodeling, 16S rRNA gene sequencing was used to the detection of gut microbiota profiling and gas chromatography was used to measure the butyrate level in mice feces. Our results indicated mid-adult mice already present distinctive gut microbiota profiling compared with young mice, concomitant with which are the lower brain dopamine level and disrupted brown adipose remodeling. KSLP treatment improved the host healthy and regulated gut microbiota with enriched Firmicutes at the expense of Bacteroidetes, particularly increased the relative abundance of bacteria functionally related to dopamine and butyrate productions, which suggest KSLP treatment constructs a healthier gut environment. In conclusion, modulation of gut microbiota and butyrate may connectively regulate dopamine production and BAT remodeling through gut-brain axis and gut-metabolism axis.

[KEY WORDS] Mid-adult; Gut microbiota; Host health; Gut-brain axis; Gut-metabolism axis
[CLC Number] R965 **[Document code]** A **[Article ID]** 2095-6975(2020)02-0090-13

Introduction

Aging is defined as "the regression of physiological function accompanied by the development of age" [1]. Alternations of a range of biological pathways [2-3] and various diseases occur in the process of aging, such as metabolic disorders [4], inflammation [5], gut dysbiosis [6] and decreased learning and memory [7], etc. Nowadays, with the improvement of healthcare, people's life expectancy has increased significantly. It is estimated that within 50 years, about 20% of the world's population will be defined as elderly (> 65 years) [8]. Novel strategies are desired for the preventing of age-associated diseases and the extending of healthy life years in the rapidly expanding aging population, which may eventually reduce the socio-economic cost of healthcare in the aging population [9].

It has long been recognized that there exists deterioration in gut-related functions that affects the gut microbiota during aging [10]. Furthermore, ever since 2008, covered by NIH launched Human Microbiome Project [11], high-throughput 16S rRNA gene sequencing technique has been extensively used in revealing the associations between gut microbial ecology and various diseases including aging. As a result, the overall structural feature as well as specific bacteria were identified for elders, which are considerably different from

[Received on] 27-Aug.-2019
[Research funding] This work was supported by the Science and Technology Department of Zhejiang Province, China (Nos. 2018C02048 and 2018F10076), and the Agricultural and Social Development Department of Hangzhou City, China (2018).
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These authors have no conflict of interest to declare.

that of younger adults [12]. The elderly gut composition tends to present an overall low diversity state, relative lower abundance of phylum Firmicutes [13-16], Clostridium cluster IV or higher abundance of Bacteroidetes [17]. Besides, low diversity has been found to associate with increased health risks [18]. These findings lay the framework for strategic interventions of maintaining a healthy gut microecology to a reduced incidence of disease and result in longevity. Nevertheless, till now, the signs of gut microbiota in the process of aging as well as its association with host healthy are not well understood.

Many approaches have been applied in attempting to maintain homeostasis and diversity of the gut microbiota i.e. the administration of pre/probiotics, intake of a fiber-rich diet and indigestible carbohydrates, or fecal microbiota transplants [19]. Specifically, herbal formula can promote the health of the human body by selectively inhibiting or promoting the growth of specific intestinal microorganisms to regulate the composition of the gut microbiota or the metabolic status [20]. Furthermore, gut microbiota has a biotransformation effect on the active ingredients from traditional herb, and to improve the bioavailability and activity of the active ingredients from herbal formula [21-22]. *Kang Shuai Lao Pian* (KSLP) is a famous traditional Chinese medicine formulated from a court prescription of the Ming Dynasty. It contains *Panax ginseng*, *Rehmannia glutinosa*, *Asparagus cochinchinensis*, *Ophiopogon japonicus*, *Lycium chinense*, *Poria cocos*. In China, it is widely accepted as a health care product for delaying senescence. There are experimental evidences showing that KSLP can improve the learning and memory ability, inhibit brain lipid peroxidation in D-galactose induced aged rats [23]. By utilizing KSLP as a tool of intervention, we are likely to reveal the connections of gut microbiota associated with host healthy in the aging process.

The aim of this work is to establish the profiling of mid-adult cecal microbiota associated with host healthy. In this regard, we investigated the associations among age, neuron dopamine, BAT remodeling, butyrate and gut microbiota in Young mice, Mid-adult mice in absence or presence of KSLP intervention. The novel links among gut microbiota and dopaminergic signaling and adipose tissue metabolism were also discussed.

Materials and Methods

Animals

Sex differences in gut microbiota composition are existed in human and experimental animals [24-25]. In current study, we only investigated the effect of KSLP on mid-adult female mice. C57/BL6 mice (female, 6-weeks or 8-months) were purchased from the Shanghai Laboratory Animal Company (SLAC, Shanghai, China) and maintained in the following conditions: 24 –26 °C, 40%–60% humidity, 12-h light/dark cycle, with food and water at will. All of the animal experiments were approved by the Animal Care and Use Committee of Zhejiang University (approved in March 2017) and followed the Guide for the Care and Use of Laboratory Animals [26].

KSLP preparation

KSLP (Batch number: 15075) was provided by Chiatai Qingchunbao Pharmaceutical Co., Ltd. (Hangzhou, China). KSLP formula mainly includes six kinds of traditional Chinese medicine: *Panax ginseng*, *Rehmannia glutinosa*, *Asparagus cochinchinensis*, *Ophiopogon japonicus*, *Lycium chinense*, *Poria cocos*. The concrete preparation method of KSLP was described in the patent (CN 1943707 B) [27]. KSLP was dissolved in Milli-Q water and prepared as drug resuspension before treating mice.

Experiment design

Mice were divided into three groups: Young (6-weeks, n = 6), Mid-adult (8-months, n = 6), Treated Mid-adult (8-months-K, n = 6). Mice were supplied with either KSLP resuspension at 0.45 $g \cdot kg^{-1} \cdot d^{-1}$ or same amount of water by intragastric administration, respectively. Six weeks later, mice were euthanatized, then cecal feces, brain, inguinal white adipose tissue (iWAT) and interscapular brown adipose tissue (iBAT) were collected. Cecal feces were stored at −80 °C till samples were sent for 16S rRNA gene sequence analysis. Tissues of brain, iWAT and iBAT were weighted and stored at −80 °C for further analyses.

In another similar setting of experiment, mice were divided into four groups (n = 5 per group). In addition to Young (6-weeks), Mid-adult (8-months), Treated Mid-adult (8-months-KSLP) groups, a group of Treated Young (6-weeks-KSLP) was also included. In the end of experiment, the total intestinal feces of mice were collected for the detection of butyrate.

Brain dopamine production

Frozen whole brain samples were used for Enzyme-linked immunosorbent assays to measure the concentration of dopamine (DA) according to the manufacturer's instructions (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China. https://www.mlbio.cn/). In brief, brain tissues were weighted, minced in to small pieces and homogenized with PBS. After spin centrifugation, clarified homogenate from each sample was added into each well of a DA antibody coated 96-well plate in duplicates. Equal mount of a series of standards were added for the construction of a standard curving. After reading the optical density (*OD*) value at 450 nm, the concentration of DA was calculated according to the standard curve and expressed as $pg \cdot g^{-1}$.

BAT remodeling

Both iWAT and iBAT were carefully dissected from both sides of the mouse and weighted. The ratio of iWAT versus iBAT (iWAT/iBAT) was calculated and considered as a sign of BAT remodeling.

Gut microbiota sequencing

Fecal samples were sent to Zhejiang Tianke high-tech Co., Ltd. (Hangzhou, China. http://www.tkgeneclub.com/tkgeneclub/index.html) for 16S rRNA gene sequencing. DNA extraction and 16S rRNA gene sequencing was done using EMP standard protocols (http://www.earthmicrobiome.org/

protocols and standards/16s) [28]. Briefly, DNA was extracted using a fecal DNA isolation kit (MoBio Laboratories, USA). For each cecal fecal sample, amplicon PCR was performed on the V3-V4 region of the 16S rRNA gene using the primer pair. The primer pairs with the barcode consisted of the forward primer (341F: 5′-CCTAYGGGRBGCASCAG-3′) and the reverse primer (806R: 5′-GGACTACNNGGGTATCTAA T-3′). All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). PCR products were mixed in equidensity ratios. Then, the mixture of PCR products was purified with Qiagen Gel Extraction Kit (Qiagen, Germany). The purified amplicons were pooled in equal concentrations for sequencing on the Illumina HiSeq2500 sequencing platform and 250 bp paired-end reads were generated. Raw sequencing data are available at Sequence Read Archive (SRA) database of NCBI and connected to bioproject PRJNA533516.

Bioinformatic analysis

Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Paired-end reads were merged using FLASH (V1.2.11, http://ccb.jhu.edu/software/FLASH/) [29], and quality-filtered using QIIME (version 1.9.1, http://qiime.org/index.html), with the criteria followed as published paper [30]. Operational Taxonomic Units (OTUs) were clustered with 97% similarity cutoff using UPARSE (Uparse V8.1.1861, http://drive5.com/uparse/) [31]. Representative sequence for each OTU was screened for further annotation by GreenGene Database (http://greengenes.lbl.gov/cgi-bin/nphindex.cgi) and RDP Classifer (http://rdp.cme.msu.edu/) against the SILVA reference database (http://www.arb-silva.de/) [32] by uclust (http://drive5.com/ usearch/manual/uclust_algo.html) [33] at 90% threshold. Non-Metric Multi-Dimensional Scaling (NMDS) analysis was displayed by vegan package in *R* software (Version 3.2.2). Unweighted Pair-group Method with Arithmetic Means (UPGMA) Clustering analysis based on weighted UniFrac distance was conducted by QIIME software (Version 1.9.1). The Shannon index, Simpson index, rarefaction and rank abundance were calculated with QIIME (Version 1.9.1) and displayed with *R* software (Version 3.2.2). Shannon index and Simpson index used *t* test for statistical significance comparison among groups. Significant differences in phylum and genus levels between groups were calculated using *R* software for *t* test to obtain *P*-values. Linear discriminant analysis effect size (LEfSe) (http://huttenhower.sph.harvard.edu/galaxy) combined the standard tests (Kruskal-Wallis sum-rank test and Wilcoxon rank-sum test) with linear discriminate analysis for statistical significance comparison.

Gut butyrate production

A portion of fecal samples (0.35 g) from the total intestinal was mixed with 1 mL of MiLi-Q water. After the sample was acidified to pH 2–3 with concentrated hydrochloric acid, 2 mL ether was added to extract butyrate. After shocked and resuspended, the sample was then centrifuged at 4000 r·min^{-1} for 10 min at 4 °C, and the upper ether solution was retained for gas chromatography measurement. An Agilent Technologies 7890A system equipped with a DB624 Capillary column and a Flame Ionization Detector was applied. The injection volume was 1 μL. The temperature program of the column oven was set as initializing at 80 °C for 5 min, rising at a ramp rate of 20 °C·min^{-1} till 200 °C, and maintaining for 5 min. Nitrogen was used as carrier gas at a flow rate of 4.0 mL·min^{-1}. The temperatures of FID and inlet were maintained at 280 and 250 °C, respectively. The concentration of butyrate was calculated according to its calibration curve and expressed as mg·g^{-1}.

Statistical analysis

Data were expressed as means ± standard errors of the means (SEM) unless otherwise indicated. Significant differences between groups were detected by multi-factor analysis of variance (one-way ANOVA or two-way ANOVA) using GraphPad Prism 7.0 software and $P < 0.05$ indicated a statistically significant difference.

Results

Impact of aging on brain dopamine production and the effect of KSLP supplement

Deficiency in learning, memory and cognitive ability is one of the significant features of aging [34]. With chronological aging, progressive loss of dopaminergic neurons in the brain and decreased dopamine secretion not only result in a decline in learning and cognitive function, but also disrupt link between the thalamus and motor cortex, leading to bradykinesia [35]. Dopamine is a key neuron transmitter in the dopaminergic signaling [36]. Regulating brain dopamine production may ameliorate the risk of neurodegenerative diseases and improve learning and cognition functions [37-38]. Previous reports implied that there exist age-related decline in dopamine synthesis and dopamine receptor density in the brain [39-41]. In the first panel of experiments, we evaluated the impact of aging and KSLP supplement on mid-adult mice brain dopamine production. ELSIA was used to detect the dopamine level. As shown in Fig. 1, compare to Young mice, Mid-adult mice have declined level of brain dopamine production (382.7 ± 24.9 *vs* 596.8 ± 29.1 pg·g^{-1}, $P < 0.01$). In current study, we did not see any behavior changes of Mid-adult mice, which is reasonable since the appearance of age-related changes in cognitive flexibility require further loss of dopamine [35, 42]. Notably, while KSLP was applied to Mid-adult mice for 6 weeks, the overall brain dopamine level increased significantly (511.6 ± 6.08 *vs* 382.7 ± 24.9 pg·g^{-1}, $P < 0.05$).

Impact of aging on brown adipose tissue remodeling and the effect of KSLP supplement

Accumulation of adipose is another frequent occurrence during aging process. In general, there exist two different kinds of adipose tissue, the white adipose tissue (WAT) that is a reservoir for lipids storage, and the brown adipose tissue (BAT) that has the ability to maintain body temperature by

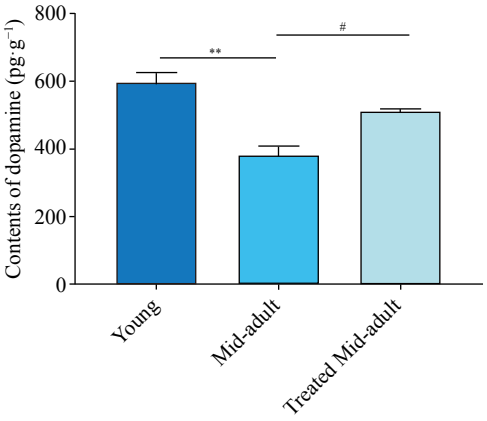

Fig. 1 KSLP supplement increases brain dopamine production in mid-adult mice. Dopamine level in per gram of brain tissue. Data were expressed as the means ± SEM (n = 3). One-way ANOVA was used to analyze statistical difference among groups. $^{**}P < 0.01$ *vs* Young group; $^{\#}P < 0.05$ *vs* Mid-adult group

producing heat [43]. A progressive age-dependent loss of WAT browning along with BAT morphological changes might happen in aged C57BL/6 mice [44]. Although how the BAT is regulated in aging process is not fully understood, recent study revealed the importance of BAT in improving metabolism and establish healthful aging [45]. In this regard, we evaluated the impact of aging and KSLP supplement on mid-adult mice body weight and adipose tissue distribution. The body weight of each mouse was measured weekly. As shown in Fig. 2A, during the experimental period, the body weight of Young mice kept increasing but that of Mid-adult mice kept at a similar level. The application of KSLP to Mid-adult mice had no effect on the body weight (Fig 2A). In the end of the experiment, the iWAT and iBAT were carefully dissected from both sides of the mice and weighted. The sum weight of iWAT and iBAT (iWAT + iBAT) and the ratio of iWAT versus iBAT (iWAT/iBAT) were calculated respectively. As shown in Fig. 2B, compared with Young mice, the overall weight of adipose tissue in Mid-adult mice increased (0.397 ± 0.058 *vs* 0.196 ± 0.017 g, $P < 0.01$); the application of KSLP to Mid-adult mice resulted in reduced amount of iWAT + iBAT, although with no statistical significance (0.313 ± 0.020 *vs* 0.397 ± 0.058 g, $P > 0.05$). The decreased overall mass of adipose tissue in Treated Mid-adult mice was due to the decrease of iWAT mass (Suppl. Fig 1A) while iBAT kept unchanged (Suppl. Fig 1B). Besides, compared with Young mice, the ratio of iWAT/iBAT was significantly higher in Mid-adult mice (4.22 ± 0.40 *vs* 1.28 ± 0.07, $P < 0.01$), and the KSLP supplement on Mid-adult mice brought back the ra-

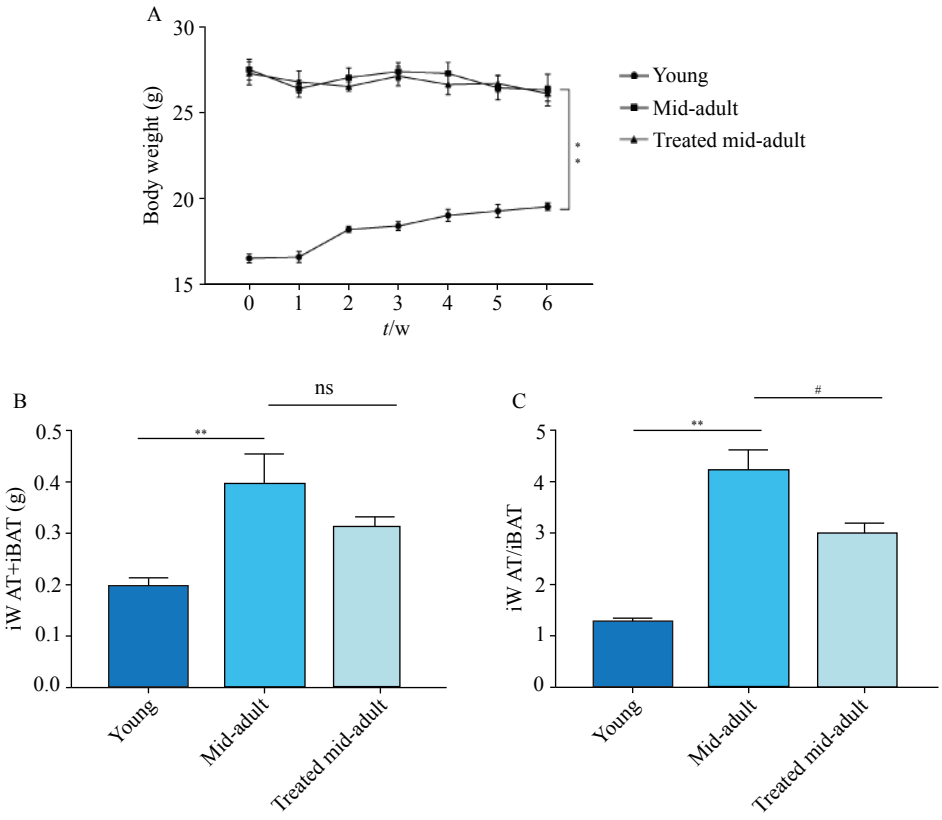

Fig. 2 KSLP supplement decreases the ratio of iWAT/iBAT in mid-adult mice. (A) The changes of body weight during the experimental course. (B) The overall mass of iWAT + iBAT. (C) Ratio of iWAT/iBAT. Data were expressed as the means ± SEM (n = 6). One-way ANOVA was used to analyze statistical difference among groups. $^{**}P < 0.01$ *vs* Young group; $^{\#}P < 0.05$ *vs* Mid-adult group; ns: no significant difference

tio of iWAT / iBAT significantly (2.99 ± 0.21 *vs* 4.22 ± 0.40, $P < 0.05$) (Fig. 2C).

It is now becoming clear that BAT does affect the development and progression of age-related diseases and could be consider as a target for anti-aging intervention [46]. In current study, we observed increased ratio of iWAT/iBAT in Mid-adult mice, but unaltered BAT mass. Our results is in accordance to Goncalves LF *et al*. who found that BAT remodeling started at 6 months while BAT mass kept unaltered until 12 months [44]. Of note, KSLP treatment contributed to the decreased ratio of iWAT/iBAT, which means that KSLP increased the relative contents of iBAT known for beneficial to body. Mid-adult mice have already shown signatures of adipose metabolic disorders, KSLP significantly downregulated the ratio of iWAT/iBAT in Mid-adult mice suggesting that KSLP has a regulatory effect on BAT remodeling ($P < 0.05$).

Aging related overall structural modulation of gut microbiota and effect of KSLP

Next, we evaluated age-related gut microbiota alterations, as well as the effect of KSLP supplement by surveying the fecal gut microbiota composition via 16S rRNA gene sequencing. A total of 857 378 high-quality and classifiable reads were generated from all samples, with an average of 47 632 ± 5138 reads per sample (Suppl. Table 1). Based on the sequence identity of greater than 97%, an average of 513 ± 116 Operational Taxonomic Units (OTUs) was consisted in each sample (Suppl. Table 1). Rarefaction curve and rank abundance analysis indicated that the sequencing depth covered rare new phylotypes and most of the diversity (Suppl. Fig 2).

Non-Metric Multi-Dimensional Scaling (NMDS) showed a significant separations of microbiota composition for each group (Fig. 3A). Furthermore, UPGMA clustering analysis based on weighted UniFrac distance was conducted, which indicated a distinct clustering of the microbiota among Young, Mid-adult, Treated Mid-adult groups (Fig. 3B). Both of the results suggest Young, Mid-adult, Treated Mid-adult mice have different gut microbiota community structures. Taken together, both aging and KSLP treatment have significant regulatory effects on the overall structure of the gut microbiota in Mid-adult mice. Notably, KSLP did not re-construct the microbiota composition to that similar to Young mice, but rather to another distinctive structure. In the following analysis, we emphasized on analyzing the alternations KSLP brought for Mid-adult mice.

KSLP supplement increases gut microbiota diversity and evenness in mid-adult mice

A declined diversity has been recognized as a predicable sign standing for an age-related gut microecology disorders associated with increased frailty [6]. In current study, Shannon index and Simpson index were utilized to compare the gut microbiota evenness and diversity. As indicated in Figs. 4A and 4B, the median value of Shannon and Simpson indexes in Mid-adult group were both lower than those in Young groups, but with no significant changes. However, Shannon and Simpson indexes were significantly increased in the Treated Mid-adult group compared with Mid-adult group. Since it is generally accepted that a higher microbiota diversity is correlated with being healthier [47], our result indicated that KSLP supplement is benefit for building up a healthier gut condition in the aspects of gut microbiota diversity and evenness.

KSLP supplement alters gut microbiota community composition at phylum level in mid-adult mice

According to the relative abundance at phylum level (Fig. 3B), Bacteroidetes, Firmicutes constituted dominant bacterial phyla in the cecal microbiota population of mice. Following this line, we calculated the mean of relative abundances of Bacteroidetes and Firmicutes among the three groups. As indicated in Fig. 5A, the mean relative abundance of Bacteroidetes were 63.41%, 69.47%, 45.17%, of Firmicutes were 29.12%, 26.91%, 48.30% in Young, Mid-adult, Treated Mid-adult group mice, respectively. Notably, the application of KSLP significantly increased relative abundance of Firmicutes and decreased that of Bacteroides. Since Firmicutes phylum have pronounced capacities to harvest nutrition from the diet over Bacteroides [48], our result implied that KSLP supplement is benefit for building up a healthier gut condition in the aspect of nutrition harvesting.

Moreover, *t*-test was used to find the significant phylum between Mid-adult, Treated Mid-adult groups. As indicated in Fig. 5B, Bacteroidetes listed at the top inhibitory phylum whilst Firmicutes listed at the top of inducible phylum responding to KSLP. It is likely that KSLP can increase the relative abundance of Firmicutes at the expense of Bacteroidetes and may help for nutrition absorption for mid-adult mice thereof.

Key phylotypes responding to the KSLP treatment in 8-months mice

To identify the specific bacteria which were characteristic among the three groups, LEfSe analysis was performed. As shown in Fig. 6A, 23 discriminative features were identified (linear discriminant analysis score > 4). Refer to the top three ranked bacterial taxa, Young mice were enriched with g_Lanchnoclostridium, f_Helicobacteraceae and c_Campylobacteria; Mid-adult mice were enriched with p_Bacteroidetes and its members of c_Bacteroidia and o_Bacteroidales; Treated Mid-adult mice were enriched with p_Firmicutes and its members of c_Clostridia and o_Clostridiales. Meanwhile, taxonomic cladogram from LEfSe analysis manifested p_Proteobacteria, p_Bacteroidetes, p_Firmicutes stood for Young, Mid-adult, Treated Mid-adult groups, respectively (Fig. 6B). Our LEfSe results were consistent with previous phylum level analysis that KSLP has an inducible regulatory effect on Firmicutes at the expense of Bacteroidetes. In addition, it suggests that c_Clostridia plays a priority role in regulating gut function during KSLP treatment.

Moreover, *t* test was used to find the significant different genus between Young mice and Mid-adult mice as well as

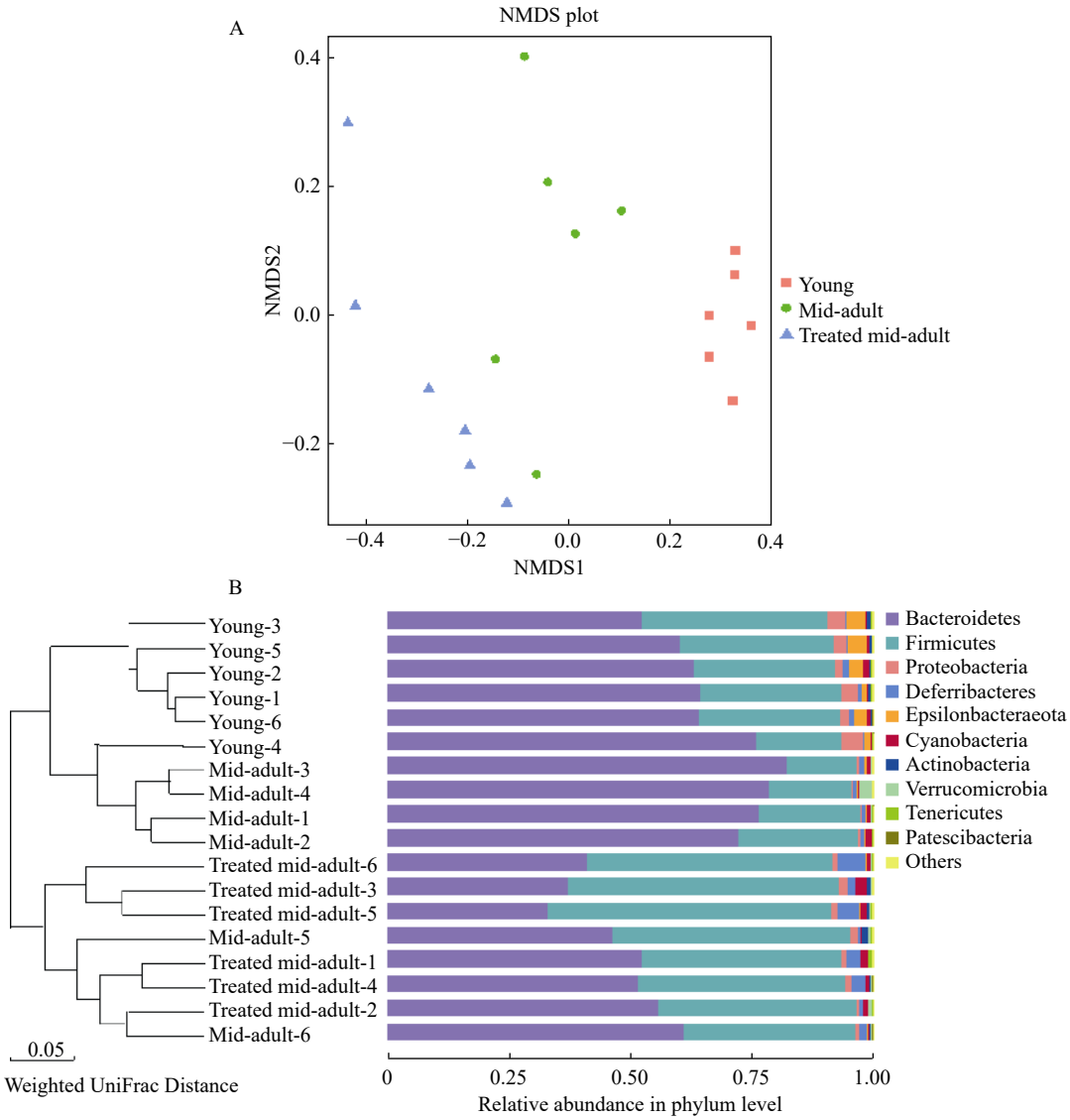

Fig. 3 KSLP supplement reconstructs bacterial community in mid-adult mice. Microbiota composition in cecal feces of Young mice, Mid-adult mice treated with or without KSLP were analyzed using 16S rRNA gene sequencing (n = 6). (A) Plots were generated using the Non-Metric Multi-Dimensional Scaling (NMDS) analysis. Young group: orange square; Mid-adult group: green circular; Treated mid-adult group: blue triangle. (B) Bacterial taxonomic profiling at the phylum level were clustered by using UPGMA based on weighted UniFrac distance.

between Mid-adult mice and Treated Mid-adult mice. Compared with Young mice, Mid adult mice present relative increased abundance of Muribaculum and Ruminococcaceae_UCG-010 whilst decreased abundance of Helicobacter, Lachnospiraceae_UCG-001, Prevotellaceae_Ga6A1_group, Parabacteroides, Anaerostipes, Lachnospiraceae_UCG-008 and Candidatus_Saccharimonas (data not shown). Compared with Mid-adult, 16 genera were identified as inducible bacteria in Treated Mid-adult group (Fig. 6C). Interestingly, amongst the 16 genera, most (13/16) belong to Clostridia class under Firmicutes phylum. Bacteria in Clostridia class are known to play crucial roles in maintaining gut homeostasis and contribute to host defense mechanisms against exogenous infections [49].Furthermore, an *in vivo* experiment revealed an association between the presence of Clostridia and increased production of norepinephrine and dopamine [50]. Those evidences support the assumption that KSLP might work through increasing Clostridia class abundance benefiting for the production of dopamine.

Besides, half of the genera (8/16) are butyrate-producing bacteria, including Lachnospiraceae_NK4A136_group [51], Anaerotruncus [52], Butyricicoccus [53], Ruminococcaceae_UCG-005 [54], Ruminococcaceae_UCG-009 [54], Lachnospiraceae_FCS020_group [51], Peptococcus [55] and Lachnospiraceae_UCG-010 [51], indicating that higher butyrate level would be exist in Treated Mid-adult group than in 8-months group.

We also observed increased relative abundance of Bilo-

phila and Oscillibacter in Treated Mid-adult group (Fig. 6C). Reduction of the abundant of Bilophila and Oscillibacter in human fecal microbiota were found in cohorts with autism spectrum disorders and patients with Crohn ’s disease, re-

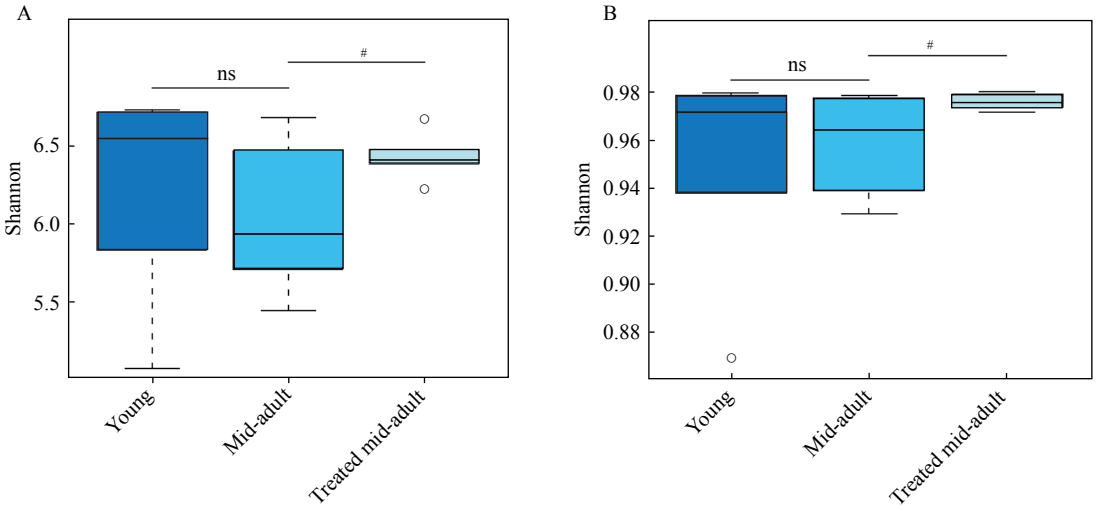

Fig. 4 KSLP supplement increases gut microbiota evenness and diversity in mid-adult mice. A. Shannon index. B. Simpson index. Data were expressed as the medians ($n = 6$). $^{\#}P < 0.05$ *vs* Mid-adult group; ns: no significant difference

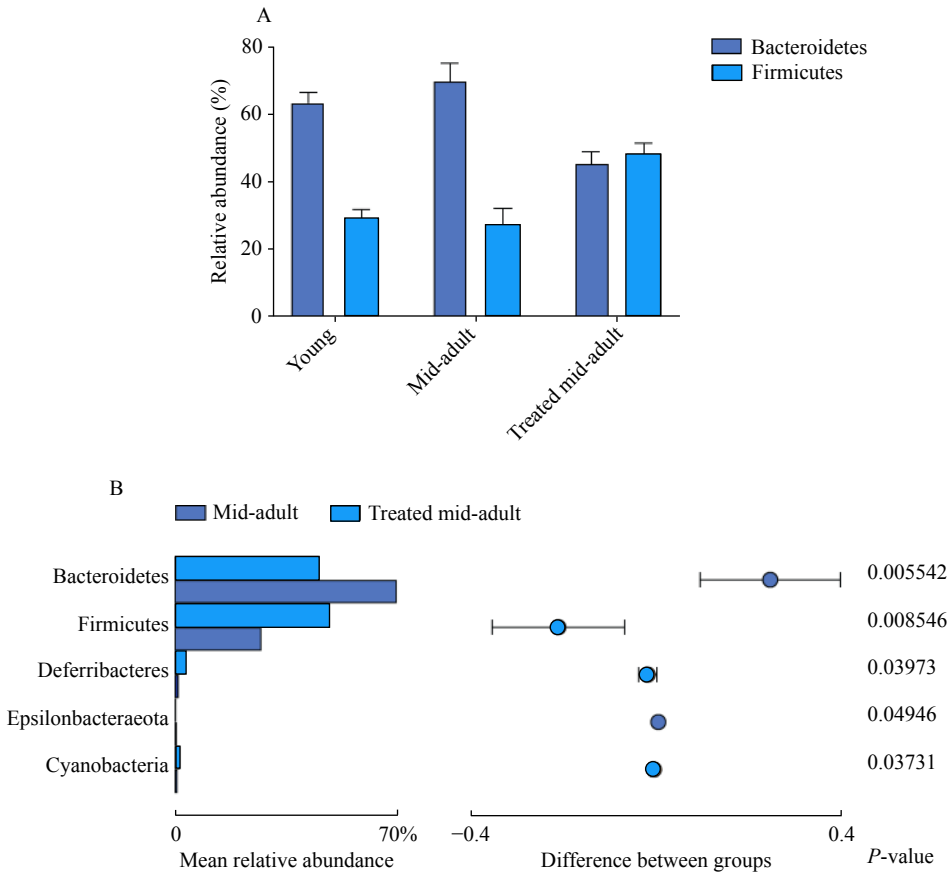

Fig. 5 KSLP supplement regulates the relative abundance of gut microbiota at phylum level. (A) Relative abundance of Bacteroidetes and Firmicutes. Data were expressed as the means ± SEM ($n = 6$). One-way ANOVA was used to analyze statistical difference among groups. (B) Significant difference phyla between mid-adult group and treated mid-adult group. The picture on the left panel showed the relative abundance of phyla, in addition, the right panel showed the difference in confidence intervals. The circular colors represented the group with a high mean. The right most of the displayed results were the inter-group significance test *P*-value for the corresponding phylum.

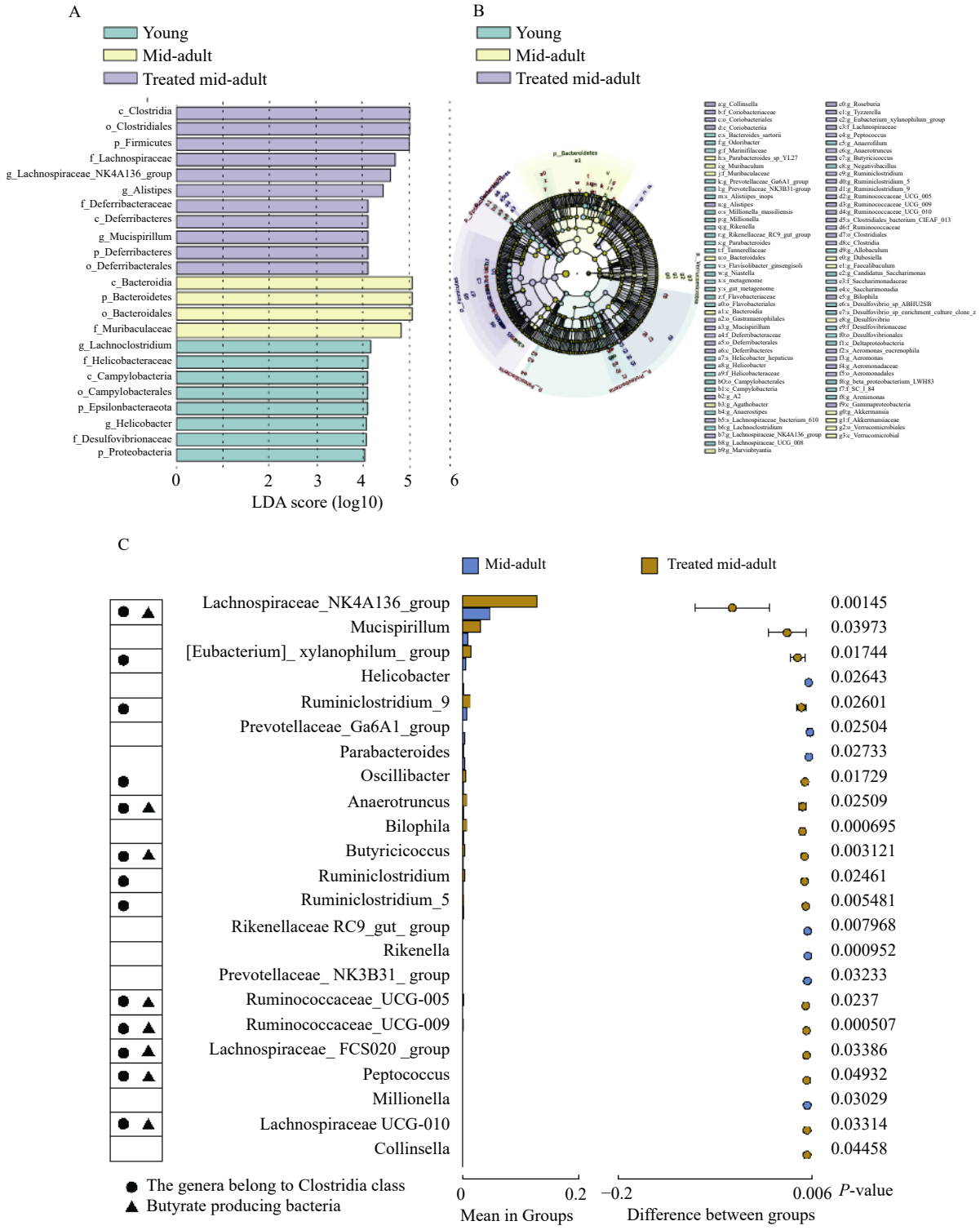

Fig. 6 Key phylotypes responding to the KSLP treatment. (A) Linear discriminant analysis (LDA) scores were computed for differentially abundant taxa in the feces microbiomes of Young (green), Mid-adult (yellow) and Treated mid-adult (purple). The LDA score indicated the effect size and ranking of each differentially abundant taxon (LDA > 4). (B) Taxonomic cladogram from LEfSe showed differences in feces taxa. Young enriched taxa (green); Mid-adult enriched taxa (yellow); Treated mid-adult enriched taxa (purple). Letters were corresponded to the right taxa. (C) Significant difference genera between Mid-adult (blue) and Treated mid-adult (brown) group. The picture on the left panel showed the mean relative abundance of genera, in addition, the right panel showed the differences in confidence intervals. The circular colors represented the group with a high mean. The right most of the displayed results were the inter-group significance test *P*-value for the corresponding genus.

spectively [56-57]. Besides, Helicobacter came out to be one of the 7 decreased genera (Fig. 6C). Helicobacter bacteria is a bacteria genus living mostly in the upper gastrointestinal tract which has been considered to be infectious and pathogenic. It was significantly reduced by calorie restriction [58].

Both age and KSLP supplement affect gut butyrate production in mice

Short chain fatty acids (SCFAs) are important metabolites produced by gut microbiota through fermenting high-fiber food or indigestible carbohydrates. Amongst the various SCFAs of acetate, propionate, and butyrate, butyrate stands out for its key role in maintain gut health [59-60]. It is well acknowledged that butyrate sustains gut homeostasis and epithelial cell integrity, acts as a source of energy for colonocytes, and interferes with pro-inflammatory signals such as NF-κB [61-62]. In elderly, reduced production of butyrate is common [6]. Evidenced from the gut microbiota analysis, our results showed that the abundance of butyrate-producing genera were increased in 8-months mice with KSLP supplement. To verify this finding, we employed gas chromatography method to detect the content of butyrate in feces. As shown in Fig. 7, the content of butyrate in the Mid-adult mice was lower than Young mice. The supplementation of KSLP not only increased the contents of butyrate in the Mid-adult mice but also in Young mice. Two-way ANOVA analysis indicated that both age and KSLP were related to the production of butyrate in feces ($P < 0.05$). KSLP supplement strengthens the population of butyrate-producing bacteria and increases butyrate in feces, therefore is responsible for a healthful aging related gut environment.

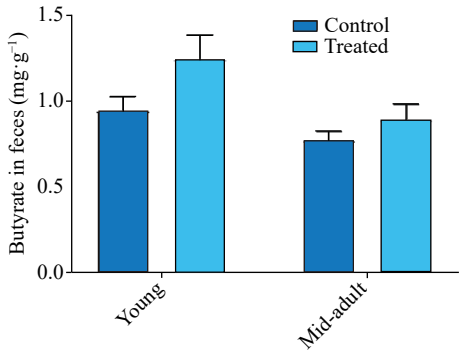

Fig. 7 Both KSLP supplement and aging affect the gut butyrate production in feces. Contents of butyrate in per gram of feces. Data were expressed as the means ± SEM (n = 5).

Discussion

We conducted our study with a group of young mice (6 weeks of age) and mid-adult mice (8 months of age) with or without traditional herbal formula KSLP by utilizing gut microbiome analysis and biochemical assays. We reported KSLP regulates the alternations in brain dopamine production, BAT remodeling, as well as gut microbiota composition changes in mid-adult mice. The key phylotypes responding to the KSLP treatment are featured by increased abundance of the phylum Firmicutes, in particular inducible bacteria that are functionally related to dopamine and butyrate production. The pharmaceutical effect of KSLP on mid-adult mice, that is brain dopamine production, BAT remodeling, and gut microbiota composition, suggests the regulation of those seemingly inter-connected systems.

Emerging evidences indicate that reduced dopaminergic signaling as well as decreased BAT activity may occur in an earlier time point through life-span, in advance of the appearance of age-associated disorders [63-64]. In current study, we showed that comparing to young mice, mice at the age of 8-months (comparable of middle adulthood person) present a relative low level of dopamine in brain, an elevated value of iWAT/iBAT, both of which could be considered as predicable leading signs for age-related cognitive deficiency and metabolic disorders. The regulatory effect of KSLP for brain dopamine production and BAT remodeling ensures the applicability for this traditional herbal formula in alleviating age-related disorders. Meanwhile, increased dopamine production provide a reasonable explanation for the previous report that KSLP can improve the learning and cognition function of D-galactose induced aging rats [23].

There is no a time point at which the gut microbiota composition suddenly alters, rather, changes occur during aging process [47, 65]. In current study, we revealed that comparing with Young mice, Mid-adult mice and Treated Mid-adult mice present altered and distinctive overall gut microbiota structural compositions. Mid-adult mice showed relatively low gut microbiota diversity, which is one of the predicable signs standing for an age-related gut microecology disorders [66]. KSLP effectively increased the diversity, meanwhile increased the relative abundance of Firmicutes at the expense of Bacteroidetes. Notably, the application of KSLP changed the overall structure of Mid-adult mice but not similar to Young mice, which is possibly matching to the assumption that pharmaceutical intervention works through regulating the functional rather than compositional change of gut microbiota [67]. Further analysis for the regulatory effect of KSLP on gut microbiota composition suggested that the abundance of many dopamine- and butyrate-producing genera are increased. As illustrated in the result section, these alterations indicated that KSLP can enhance the digestion and absorption function of mice and can lead to the increased production of butyrate and dopamine, therefore helps to build a better gut environment, eventually lead to the delaying of age-related disorders. As an approval of relevant experiment, the production of butyrate is accumulated in Treated Mid-adult mice gut in deed.

The existing of gut-brain axis may interpret the novel link between the effects of KSLP regrading gut microbiota compositional changes and brain dopamine production. The crosstalk between gut and brain appears in several aspects: 1) stimulation of vagal afferent fibers. Previous researches revealed that stimulation of vagal afferent fibers from the upper intestinal tract causes dopamine release in the brain,

the process of which are sensitive to changes of gut microbiota [68-69]. 2) translocation of gut hormones. It is known that in addition to the bacterial metabolites like SCFAs, gut also produces hormones including DA, γ-aminobutyric acid (GABA), serotonin (5-HT) and noradrenaline (NA), all of which can be translocated to the brain [68]. 3) dopamine-producing enzymes. It was recently revealed that the gut-brain axis is responsible for the synthesis of dopamine-producing enzymes in the brain [70]. In current study, we showed that KSLP changed gut microbiota in Treated Mid-adult mice, especially increased the abundance of Clostridium bacteria that are functionally related to dopamine production in gut, those changes may eventually lead to the increased production of dopamine in the brain.

Gut microbiota has long been recognized as an environmental factor contributes to the regulation of energy harvest and fat storage [48, 71]. Indeed, it is recently reported that herbal medicine ganoderma lucidum presents therapeutic effect against high-fat diet inducing-obesity is actually working through the regulating of gut microbiota composition [72-73]. Previous researches revealed both higher gut microbiota diversity and higher butyrate content are capable of reducing fat storage through activating enzymes related to fatty acid metabolism [74-75], which indicated gut-metabolism axis as one of the causal reasons for adipose metabolism. Besides, Li Z etc. demonstrated that oral but not intravenous administrated butyric acid is able to activate BAT, and that the effect of which could be abolished by subdiaphragmatic vagotomy. Therefore, the author concluded that butyric acid can activate BAT through the gut-brain axis [76]. In current study, we showed that KSLP increased the gut microbiota diversity and butyrate production in Mid-adult mice, it is likely that both gut-metabolism axis and gut-brain axis account for the BAT remodeling effect of KSLP.

It remains open for which components in KSLP are potentially responsible to the alteration of gut microbiota and butyrate. Recent researches proved that although exhibit poor oral bioavailability, many herbals and herbal formulas administrated orally are still able to improve the gut microbiota dysbiosis in diseased human cohorts and model animals [77]. It is believed after oral administration, the herbal components especially which are difficult to be absorbed by intestine reach the colon where most gut microbiota settle; those unabsorbed herbal components interact with gut microbiota, thus modulate the composition of gut microbiota [78]. Plant-derived polysaccharide is recognized as one of those unabsorbed components which can interact with gut microbiota [78]. KSLP is an herbal formula including *Panax ginseng*, *Rehmannia glutinosa*, *Asparagus cochinchinensis*, *Ophiopogon japonicus*, *Lycium chinense*, *Poria cocos*, most of which contain polysaccharide components. We speculate polysaccharide component of KSLP might play an important role in modulating Mid-adult gut microbiota. As for the butyrate production, on one hand, it is likely increasing butyrate contents in feces after KSLP administration is due to the increased abundance of butyrate-producing bacteria. On the other hand, polysaccharide components can be directly metabolized by gut microbiota into SCFAs *via* certain enzymes [79-80], it is also possible that the polysaccharide component of KSLP acts substrate provider of SCFAs. Our future study will be focused on exploring whether polysaccharides are the responsible components of KSLP. Besides, whether the activities for the butyrate-producing related enzymes are alerted by KSLP is also worthy of further study.

To sum up, as graphically illustrated in Fig. 8, KSLP is able to modulate gut microbiota toward a condition featured by increased diversity, enriched relative abundance of bacteria in Firmicutes phylum at the expenses of Bacteroidetes.

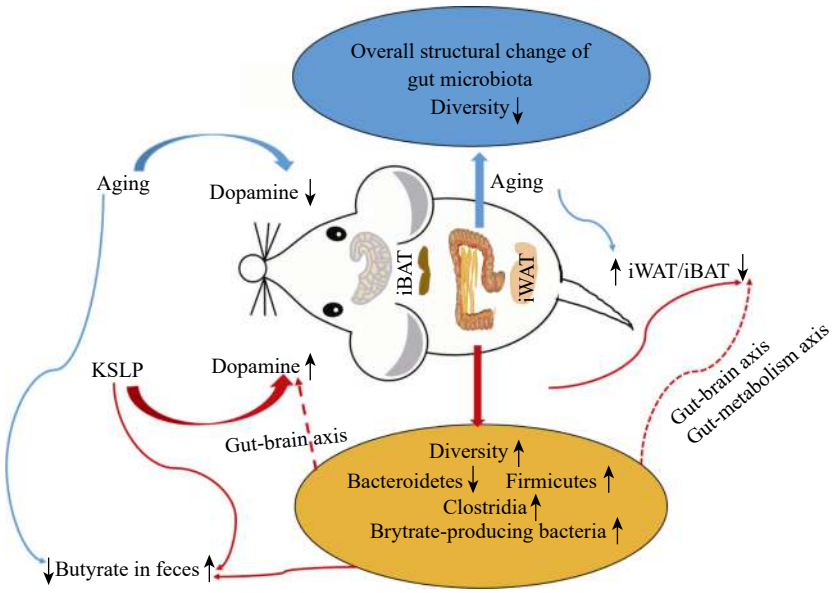

Fig. 8 The graph demonstrates the overall alternations of Mid-adult mice and the effects of KSLP.

The alterations of gut microbiota composition and increased Clostridia class abundance may account for increased dopamine in brain likely through the existing gut-brain axis. Meanwhile, increased gut microbiota diversity and butyrate production may account for the BAT remodeling probably through the gut-metabolism axis and gut-brain axis.

Conclusions

Altogether, the presented results implied that disrupted brain dopamine production and BAT remodeling are predicable leading signs for age-related cognitive deficiency and metabolic disorders. Regulating of gut microbiota to an increased diversity and the phylotypes with enriched abundance of the Firmicutes phylum and in particular inducible genera functionally related to dopamine and butyrate production can be considered as profiling of the mid-adult cecal microbiota associated with host healthy. Our current studies are helpful for a better understanding of the correlations among the aging process, gut microbiota, cognitive function and adipose metabolism, eventually from which contribute to the discovery of intervention targets for a healthy aging.

Supplementary materials

Supporting information of this paper can be requested by sending e-mails to the corresponding authors.

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Cite this article as: GONG Shu-Qing, YE Ting-Ting, WANG Mei-Xia, HONG Zhu-Ping, LIU Li, CHEN Huan, QIAN Jing. Profiling the mid-adult cecal microbiota associated with host healthy by using herbal formula *Kang Shuai Lao Pian* treated mid-adult mice [J]. *Chin J Nat Med*, 2020, **18**(2): 90-102.

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