Title : The Effect of Fermented Milk Containing *Lactobacillus casei* Strain Shirota on Sarcopenia in Elderly Taiwanese: Interactions with the Nutrients Utilization, Diversity of Gut Microbiota, Microbiota-derived Metabolites and Muscle loss

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1. Introduction

Aging-associated diseases represent a growing issue in modern society due to the ever-increasing proportion of elderly individuals. Sarcopenia refers to the progressive loss of skeletal muscle mass and strength and shares many characteristics with other disease states typically associated with risks of falling and fracture, including osteoporosis, frailty, and obesity. It is a common geriatric syndrome; its prevalence has been estimated to be up to 35% in hospital wards [1, 2] . Osteoporosis, frailty, and obesity result in negative health outcomes later in life [3]. Therefore, investigating sarcopenia is an important component of ensuring 'healthy aging'.

1.1 Possible Mechanisms of Sarcopenia

Many hypothesis have been suggested to explain sarcopenia: an increase in connective tissue, muscle steatosis, impaired muscle metabolism, an increase in inflammatory markers (e.g., TNF-α and IL-6), an increased stiffness of myofibers, slower kinetics in establishing myosin-actin crossing bridges, increased oxidative stress, mitochondria dysfunction, hormonal imbalance, and decreased capillary flow [4-8]. The production of reactive oxygen and nitrogen species (RONS) and oxidative damage has long been thought to be a potential mechanism of age-related muscle atrophy via the radical theory of aging. According to this theory, RONS damage proteins, lipids, and DNA, leading to tissue dysfunction [9] . Moreover, an essential role has been proposed for RONS in regulating the IGF-AKT-mTOR signaling pathway [10] , which, in turn, can directly impact control of muscle protein turnover. The contribution of RONS to age-related muscle decline remains debatable, and the use of antioxidant therapies has proven to be largely unsuccessful [11]. Antioxidants potentially have adverse effects on muscle [12]. As such, while their role is difficult to define in humans there is little evidence that redox imbalances are key drivers of either age-related muscle atrophy or human disuse atrophy [13].

1.2 Aging Gut Microbiota

Gut microbiota constitute a large, diverse, and dynamic ecosystem that generates cross talk between the gut and host. It can communicate with the host by modulating the gut-brain axis, which is a bidirectional neurohumoral communication system between the gut and brain. The gut-brain axis has now been expanded to the microbiota-gut-brain axis [14]. A growing body of evidence suggests that the innumerable microorganisms that populate the mammalian gastrointestinal tract (gut microbiota) are tightly linked to the aging process of their host [15, 16]. Age-related

changes in the bacterial composition of microbiota are well known, and alterations of gut microbiota driven by diet may affect the health of elderly people [17, 18]. This lack of knowledge is noteworthy because aging alone alters the gut microbiota (dysbiosis) and likely contributes to age-related inflammation [19-22] , a condition often referred to as “inflammaging”[23-25]. Importantly, age-related dysbiosis may be a major contributor to the increasing prevalence of many age-related diseases and the poorer outcomes observed in the elderly after an acute injury.

1.3 Microbiota-derived Metabolites

Dietary tryptophan metabolism is a complex pathway involving either mammalian or bacterial enzymes or a combination of both. Dysregulation of this tryptophan metabolism pathway and associations with metabolic disease is better known for mammalian tryptophan derivatives. Bacterial enzymes (e.g., tryptophanase) convert dietary tryptophan into indole. Indole can be metabolized to indole-3-propionic acid (IPA) solely by bacteria. The role of these indole derivatives in metabolic disease is not known. Gut microbiota have been reported to influence tryptophan metabolism and serotonin biosynthesis[26, 27]. Another well known microbiota-derived metabolite is short chain fatty acid (SCFA). Dietary fibers are insoluble or soluble carbohydrates that are non-digestible by human digestive enzymes. They are hydrolyzed and fermented in the colon through the action of anaerobic bacteria, producing mostly SCFA. SCFA contributes to certain metabolic processes, such as satiety increase, control of lipid synthesis in liver and fat storage, increase of glucose uptake from small intestine and reduction of inflammation[28-30].

1.4 Trace elements

Trace elements play an important role in maintaining metabolic hemostasis and preventing many aged-associated diseases [31]. However, all trace elements are toxic when ingested in excess amounts, yet many elements such as potassium, manganese, and magnesium are necessary at trace levels for various bodily functions. Previous studies indicated that long-term accumulations of non-essential elements may be related to bone and muscle weakness [32-34]. Only one Korea study reported that exposure to lead, cadmium, and mercury will increase the prevalence of sarcopenia in the elderly population. This study data show that the odds ratio was increased with increasing concentration quartile of lead ($P<0.001$), mercury ($P<0.001$) and cadmium ($P<0.001$) in both genders, and the sum of the three heavy metals ($P<0.001$) in females [35]. There is a limited report to study the association between trace elements

and sarcopenia in the elderly group. Therefore, the causality between trace elements and sarcopenia should be verified in further longitudinal studies.

1.5 Regulatory T cells

Regulatory T cells (Tregs) are a crucial subset of immune cells responsible for maintaining immune system homeostasis and preventing excessive immune responses. These cells play a vital role in self-tolerance by suppressing the activity of other immune cells, thus reducing the risk of autoimmune diseases. Tregs exert their effects through the secretion of anti-inflammatory cytokines and direct interactions with other immune cells to modulate immune responses. Recent research has highlighted the significant impact of Tregs on muscle health and intestinal immunity. Studies have shown that Tregs can modulate inflammatory factors to suppress muscle inflammation, which contributes to muscle repair and regeneration. Additionally, beneficial gut microbiota can induce Tregs to prevent intestinal inflammation and inhibit the adhesion of pathogenic bacteria to the gut mucosa, thereby maintaining gut health [36, 37]. These findings underscore the essential role of Tregs in sustaining overall health and mitigating chronic diseases.

1.6 Beneficial Effects of *Lactobacillus casei* strain Shirota (LcS) on GI Function, Brain Function, and Inflammation

Lactobacillus casei strain Shirota (reclassified as *Lacticaseibacillus paracasei* strain Shirota in April 2020) (LcS) is a well-known probiotic strain that has been approved and is generally recognized as safe by the United States Food and Drug Administration. LcS has been suggested to provide health benefits by balancing gut microbiota, improving gastrointestinal dysfunction, preventing infection and cancer, and modulating inflammatory and immune responses [38]. Previous studies have demonstrated that LcS improves mood disturbances in the elderly [39] and decreases anxiety symptoms in patients with chronic fatigue syndrome [40]. In a pilot trial, LcS also suppressed the onset of physical symptoms in healthy students exposed to academic stress [41]. However, studies have not examined fully whether LcS relieves psychological stress-induced responses associated with the microbiota-gut-brain axis of healthy subjects.

1.7 Preliminary Work

In our previous work, we investigated whether LcS decelerates age-related muscle loss and its underlying mechanism with SAMP8 mice [42]. The body weight and composition, muscle strength, protein intake, mitochondrial function, reactive

oxygen species, IL-6 and GI function were examined in this study. From the result we found that LcS significantly attenuated decreases in muscle mass, holding impulse, and grip force in 28-week-old female SAMP8 mice. Compared with the control group, mice in the Sarcopenia with Fermented Milk containing *Lactobacillus casei* Strain Shirota group (LcS group) also exhibited higher mitochondrial oxygen consumption and biogenesis. Taken together, LcS may extenuated muscle lose during aging; this effect may have been caused by preservation of the mitochondrial function via the gut-muscle axis in SAMP8 mice.

2. Study Objective

We hypothesized that long-term Fermented Milk Containing LcS supplementation would improve nutrient utilization and GI function, induce Treg cell activation, and balance the inflammatory response in elderly Taiwanese individuals with sarcopenia. These changes were expected to be associated with alterations in gut microbial composition and microbial metabolites, ultimately impeding the progression of sarcopenia in this population. The primary and secondary objectives were as follows:

2.1 Primary objective

- To assess changes in gut microbiota in elderly individuals with sarcopenia given Fermented Milk Containing LcS for 12 weeks, The primary objective was to confirm the efficacy of Fermented Milk Containing LcS in restructuring the gut microbiota composition against aging-related gut dysbiosis in elderly individuals with sarcopenia.

2.2 Secondary objective

- To evaluate the efficacy of Fermented Milk Containing LcS intake in alleviating reactive oxygen species (ROS) and changing biomarkers from baseline, including TNF-α, TGF-β, IL-6, IL-10, and IL-17.
- To understand the impact of Fermented Milk Containing LcS intake on impeding the progression of muscle loss in elderly individuals with sarcopenia.
- To investigate the correlations between Fermented Milk Containing LcS intake and gut microbiota microbial metabolites, nutrient utilization states, trace elements, GI function, and immune regulation in elderly individuals with sarcopenia.

3. Study plan

3.1 Overall designs

This was an open label trial to examine the effects of Fermented Milk Containing LcS supplementation on the elderly with sarcopenia. Subjects were between 65-85 years old and either male or female. A total of 120 subjects were enrolled. The study design included the recruitment of 80 elderly individuals with sarcopenia, who were randomly assigned to either the Fermented Milk Containing LcS supplement group (LcS group) or the non-treatment group (SC group) in a 1:1 allocation ratio. Additionally, 40 elderly individuals without sarcopenia were included as healthy controls (NS group) for comparative analysis. The randomization process was conducted using a lottery method to ensure balanced group allocation. Subject participation was last 12 weeks. Subjects who were withdrawn from the study would be replaced until 40 subjects per group.

3.2 Efficacy assessment

Efficacy of Fermented Milk Containing LcS supplementation was evaluated for per-protocol (PP) analysis set population who completed the study by comparing sarcopenia groups with and without Fermented Milk Containing LcS. Comparisons were made in body composition, physical performance, gut microbiota composition, microbiota metabolites, ROS, inflammation status, anabolic hormone level, immunity evaluation, trace element level, and responses to various questionaries.

3.3 Primary and Secondary Endpoints

Primary Objective and Endpoint Alignment:

To ensure a comprehensive evaluation of Fermented Milk Containing LcS supplementation, the study defined primary and secondary endpoints based on existing literature and biological plausibility. The primary endpoint, gut microbiota composition changes, was selected due to the established role of probiotics in modulating microbial diversity and function. Gut dysbiosis has been implicated in sarcopenia, making this a crucial parameter for assessing LcS efficacy.

The secondary endpoints included oxidative stress markers (ROS levels, GPx, and CAT activity), inflammatory biomarkers (TNF-α, IL-6, IL-10, TGF-β), muscle mass and physical performance, and trace element levels. These were chosen based on the hypothesis that LcS supplementation could mitigate oxidative stress, inflammation, and heavy metal accumulation, thereby influencing sarcopenia progression. The selection of these endpoints aligns with previous findings that link gut microbiota to

metabolic and immune functions, which are critical in age-related muscle degeneration.

4. Study intervention

4.1 Study supplementation and administration

Test beverages, Yakult 300 LIGHT Fermented Milk containing LcS at more than $3x10^{10}$ CFU per 100 mL bottle, were provided by Yakult Co., Ltd. (Taipei, Taiwan) (Table 1). The beverages were sent to participant weekly (14 bottles/week) and stored at temperatures ranging from 0–10℃. The subjects in the LcS supplemented group consumed 2 bottles of the test beverages per day (104 kcal/day) at approximately the same time.

The study protocol included rigorous adherence monitoring through weekly follow-ups, where participants were required to return unused bottles and complete adherence logs. Dietary records and questionnaires were also implemented to monitor additional probiotic intake or dietary factors that could influence outcomes. Additionally, compliance biomarkers, such as changes in gut microbiota profiles, were analyzed post-intervention to validate adherence.

Table 1. Nutrition fact of Yakult 300 LIGHT Fermented Milk

Yakult 300 LIGHT Fermented Milk per bottle (100 mL)	
Ingredients	Content
Calories	52 kcal
Fat	0 g
Protein	1.3 g
Carbohydrate	14.5 g
Sugar	10.0 g
Sodium	17 mg
Calcium	60 mg
Vitamin C	28 mg
Vitamin D	0.9 μg
Vitamin E	3.4 mg
Total number of LcS	Over 3 $x10^{10}$ CFU

4.2 Restriction and withdrawal

Subjects were prohibited to use other treatments for sarcopenia and discontinued

such treatments at least 4 weeks before the screening visit. Subjects were not allowed to take the following items after screening and were withdrawn from the study if they did not comply:

1. Probiotics, except for the allocated experimental drink
2. Antibiotics
3. Vitamin D or Ca
4. Oral or injected corticosteroid
5. Drug affecting the immune status
6. Chemotherapy
7. Structured physical exercise training program

Subjects were allowed to withdraw his or her consent at any time. The subjects were monitored every 2 weeks to ensure the compliance of taking the experimental drink.

5. Subject Enrollment

The subjects were recruited from Taipei Medical University Hospital, Ministry of Health and Welfare, Taipei Municipal Wanfang Hospital (Managed by Taipei Medical University), and Community Integrated Care Service Center-Taipei Community Care (Stone Soup) in Xinyi District commissioned by Taipei Medical University. The definition of sarcopenia was based on the algorithm of Asian Working Group for Sarcopenia (AWGS).

5.1 Inclusion Criteria

1. Age between 65 and 85 years
2. No use of hormonal replacement therapy

5.2 Exclusion Criteria

1. Active cancer: currently receiving cancer treatment or have received cancer treatment within the last 3 months
2. Weight change ≥ 5% or weight change ≥ 5 kg within the past 3 months
3. BMI > 30 kg/m^2
4. Disease requiring chronic use of prescription corticosteroids
5. History of ischemic or hemorrhage stroke
6. Unstable or uncontrollable hypertension (>180/110 mmHg)
7. Doing hemodialysis or peritoneal dialysis within the last 3 months
8. Participation in a structured physical exercise training program within the past 2

year; previous use of creatinine supplementation; use of drugs that can affect bone metabolism (e.g., glucocorticoids, bisphosphonates, vitamin D or calcium).

9. Antibiotics use in the past 3 months.

10. Products of probiotic use in the past 2 weeks.

11. Living abroad for one month in the past 3 months

12. Be hospitalized or have acute disease in the past 3 months

13. Abnormal thyroid function

14. Allergic to milk

5.3 Algorithm of recruitment and grouping

Participants who passed the exclusion criteria started the algorithm of recruitment. In order to increase the efficiency of recruitment, **two stages of sarcopenia screening** had been designed. The participants who passed step 1 proceeded with the step 2 screening as follows:

5.3.1 Step 1: First Screened cases

(1) Calf circumference: Calf circumference was measured by using tape. Low calf circumference was defined as:

- Men: <34cm
- Women: <33cm

(2) Risk of sarcopenia: Risk of sarcopenia was measured by using SARC-F questionnaire. High score was defined as:

- Score: ≧4

5.3.2 Step 2: Second screened and confirm sarcopenia

(1) Skeletal muscle mass (SMI, Unit: mass (kg)/height (m^2)) was measured by using bioelectrical impedance analysis (BIA), and Low SMI was defined as

- Men: SMI <7.0 kg/m^2
- Women: SMI <5.7 kg/m^2

(2) Handgrip strength: Handgrip strength was measured by using electronic hand grip dynamometer. Low handgrip strength was defined as:

- Men: <28 kg
- Women: <18 kg

(3) Limb strength: Limb strength was measured by using Time for 5 times for chair stand method. Low limb strength was defined as:

- Time for 5 times for chair stand: ≧12s

Sarcopenia was defined by (1) and one of (2) or (3)

The participants who failed to pass the first screening were classified as **“non-sarcopenia group”**. The participants of non-sarcopenia group only underwent the baseline assessment. The participants who were successful in the screening for sarcopenia followed the randomization procedure and classified into “sarcopenia group” and “LcS group”. Randomization was performed using a lottery method to allocate participants at 1:1 ratio (Figure 1).

5.3.3 Workflow of participant's recruitment and grouping

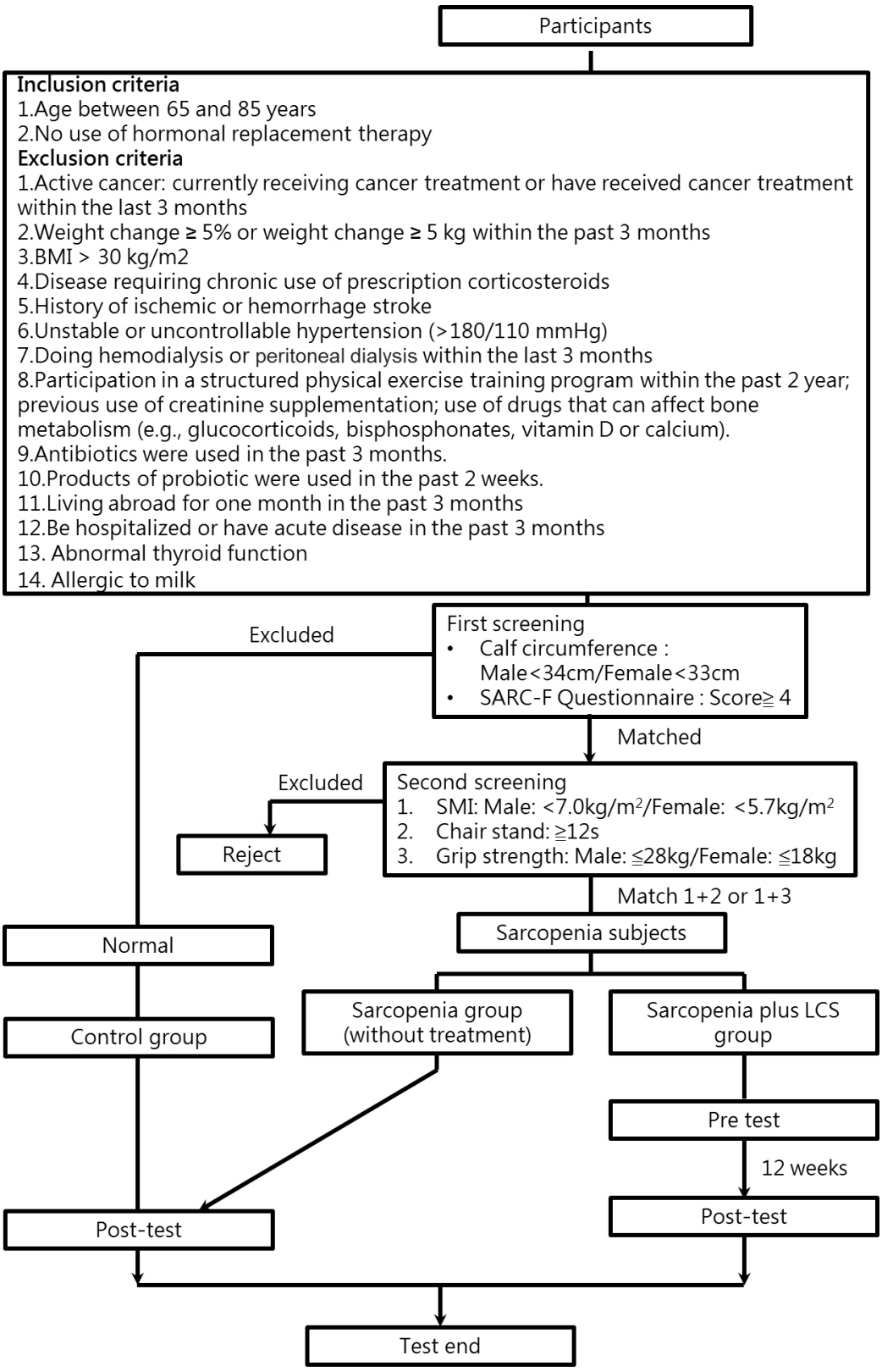


Figure 1. Workflow of participant's recruitment and grouping

6. Material and method

6.1 Demographic data and medical history

Demographic data (included basic information, nutritional assessment, food frequency ingestion, daily personal habits, physical activity and oil-smoke exposure assessment etc.) and a complete medical history were collected at the screening visit. The medical histories were traced back for 3 months. Meanwhile, the history of structured physical exercise training program was traced back to past 2 years before screening.

6.2 Anthropometric measurement and Body Composition Assessment

Anthropometric measurements, including height, weight, waist circumference, arm muscle circumference, hip circumference, and calf circumference, were determined using a measure tape and weight scale. Body composition was determined by bioelectrical impedance analysis (BIA).

6.2.1 Bioelectrical impedance analysis (BIA)

Participants stood on floor mat without shoes and socks. Arms did not touch the trunk part of body, and were spread naturally to a 15 degree angle away from trunk. The hand electrodes were marked THUMB for the thumb and MIDDLE for the middle finger. The foot electrodes were positioned between examinee's anklebone and heel. Electric current was supplied from the electrodes on the tips of the heels of both feet and the fingertips of both hands.

6.3 Muscle strength

Grip strength was measured under standard conditions with a well-studied model of a handheld dynamometer with reference populations could be a reliable surrogate for more complicated measures of muscle strength in the lower arms or legs. The measurement used electronic hand grip dynamometer. Low handgrip strength was defined as Men: <28 kg; Women: <18kg. Participants sat and stood five times, and time was calculated using a timer. Low limb strength was defined as time for 5 times chair stand: $\geqq$12s.

6.4 Physical performance

Physical performance was determined by using 6-meter usual gait speed, timed Up and Go (TUG) test, and Short Physical Performance Battery (SPPB).

6.4.1 Walk speed

Participants were instructed to walk from a standing start at a pace that was normal and comfortable for them or to walk as fast as they could until they reached

the end of the marked path. A trained examiner walked behind the participant and stopped timing when the participant's foot contacted the floor at the end of the walking course. Participants were provided rest breaks as needed throughout the testing session. Low physical performance was defined < 1 m/s.

6.4.2 TUG

Participants were instructed to stand up from the chair, walk 3 meters forward and go back to sit on the chair. Low physical performance was defined ≧ 20s.

6.4.3 SPPB

Participants were followed to SPPB to do the test. Low physical performance was defined ≦ 9 pt (Appendix Ⅰ).

6.5 Gut microbiota composition and microbiota-derived metabolite analysis

6.5.1 Fecal sample collection and bacterial genomic DNA isolation

Fresh fecal samples were collected from individual participant. Participants were instructed to place toilet paper at the front of the toilet bowl before defecation to prevent fecal contamination with urine or water. They were asked to defecate in the opposite direction of their usual practice, ensuring the feces were deposited onto the toilet paper. Using the collection stick provided in the sampling kit, participants collected fecal samples and transferred them into the provided tubes. One of the tubes contained a preservation solution, which required thorough mixing after sample collection. Both tubes were then stored in a -80 °C freezer.

The genomic DNA was extracted by using the QIAamp DNA Stool Mini kit according to the manufacturer's protocol. DNA concentrations were measured by using NanoDrop2000. The fecal samples were stored at -80℃ refrigerator.

6.5.2 16S rRNA amplicon and data analysis

The V1-V9 region of the 16S rRNA gene by PCR were referred to the Illumina 16S Metagenomic Sequencing Library Preparation protocol. The amplicons were paired and sequenced on an Illumina MiSeq 2000 platform according to manufacturer's protocol. The data was analyzed with Quantitative Insights into Microbial Ecology 2 (QIIME2) with NCBI 16S RNA database (2023/01). The UPARSE algorithm was used to align with identical sequences and determine the representative operational taxonomic unit (OTU) sequences. For sequences with 97% similarity, they were clustered as same OTU. Alpha diversity analysis (Shannon index) was performed using QIIME2 while beta diversity analysis was analyzed using weighted principal coordinate analysis (PCoA). The online Galaxy workflow

framework was used to determine the linear discriminant analysis (LDA) effect size (LEfSe).

6.5.3 Fecal Short Chain Fatty Acids (SCFAs), IPA and TMAO Determination

The fecal samples were diluted in 0.5% phosphoric acid and homogenized. After centrifuge, the supernatant was mixed with 4-ethylpentanoic acid in ethyl acetate, and kept the supernatant at -20℃.

The untargeted metabolomics was performed using HPLC coupled with a Q-Exactive Orbitrap Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) as previously described[43, 44]. Liquid chromatography was performed on HPLC system using a BEH C18 column (1.7 μM, 2.1 mm x 100 mm, Waters Corporation). The injection volume was 2 μL with a flow rate of 0.3 mL/min and the column temperature was maintained at 35℃. HPLC linear gradient conditions were: 0–0.5 min 1 % B, 0.5–2.5 min from 1 % B to 10 % B, 2.5–3.5 min 65 % B, 3.5–5.0 min from 35 % B to 100 % B, and 5.0–9 min 1 % B [solvent system A: water/formic acid (100:0.1, vol/vol); B: acetonitrile/formic acid (100:0.1, vol/vol)]. The capillary temperature and voltage (+ and -) were maintained at 275 ℃ and 3,600 and 3,200 V, respectively. The samples for quality control (QC) were prepared by mixing all the samples. Data were acquired by Thermo Data Analysis software Compound Discoverer TM2.0. The raw intensities were transformed and normalized. For multiple peaks mapped to the same metabolites, the average intensity value was used. The matrix was then subjected to principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) using the R ropls package (version 1.21.0) to obtain differential metabolites between groups.

6.6 Analysis of blood biochemical parameters

6.6.1 Whole blood, serum and plasma samples preparation

Blood samples were collected from the subjects at pre-test and post-test. Whole blood was placed in collection tubes containing K_2EDTA and then stored at 4℃ until analysis. For plasma preparation, blood samples were placed in collection tubes containing heparin and centrifuged at 3,000 *g* for 10 min then took supernatant. For serum preparation, blood samples were placed in collection tubes and then centrifuged at 3,000 *g* for 10 min then took supernatant. The plasma and serum were stored at -70℃ until analysis.

6.6.2 Biochemical parameters

All biochemical parameters were performed one day before the start of the

intervention and at the end of intervention. Albumin, prealbumin, cholesterol, HDL-C, LDL-C, triglyceride, TSH, free T4, hsCRP, HbA1c (%), fasting glucose, fasting insulin, HOMA-IR, ALT, AST, creatinine, eGFR, WBC, RBC, HGB, HCT, MCHC, platelet and RDW-CV were determined after fasting for 8 hrs. Normal values of biochemical parameters were shown in Table 2.

Table 2. Normal value of biochemical parameters

Item	Normal value
TSH (μIU/ mL)	0.35-5.50 μIU/mL
Free T4 (ng/dL)	0.76~1.64 ng/dL
hsCRP (mg/dL)	<1.0 mg/L
Albumin (g/dL)	3.5-5.5 g/dL
Pre-albumin (mg/dL)	20-40 mg/dL
CHOL (mg/dL)	<200
HDL-C (mg/dL)	M:>40 mg/dL；F:>50 mg/dL
LDL-C (mg/dL)	<100(Desirable)
TG (mg/dL)	<150 mg/dL
HbA1c (%)	4.0~5.6%
GLU(AC) (mg/dL)	70~99 mg/dl
insulin (μIU/mL)	1.5-17.0 μU/mL
HOMA-IR	HOMA-IR ≦ 1.4
GOT(AST) (U/L)	0-34 U/L
GPT(ALT) (U/L)	0-36 U/L
BUN (mg/dL)	6-21 mg/dL
CREA (mg/dL)	M:0.64~1.27 mg/dL；F:0.44~1.03 mg/dL
eGFR (mL/min/1.73 m^2)	≧ 60 mL/min/1.73 m2
WBC (1000/μl)	M:3.9-10.6 1000/μL；F:3.5-11.0 1000/μL
RBC (million/μl)	M:4.5-5.9 Million/μL；F:4.0-5.2 Million/μL
HGB (g/dL)	M:13.0~18.0 gm/dl；F:11.0~16.0 gm/dl
HCT (%)	M:41-53%；F:36-46%
MCHC (g/dL)	31-37 gHb/dL
Platelet (1000/μL)	150-400 1000/μL
RDW-CV (%)	11.5-14.5%

6.6.3 Analysis of pro-inflammatory and anti-inflammatory cytokines

Pro-inflammatory cytokines (IL-6 and TNF-α) and anti-inflammatory cytokines (IL-10, IL-17 and TGF-β) were determined by using ELISA Kits (Elabscience Biotechnology Inc) via ELISA reader (BioTek, PowerWave XS2, City, State, USA).

6.6.4 Analysis of antioxidant enzymes

Superoxide dismutase (SOD) (E-BC-K019, Elabscience Biotechnology Inc.), Glutathione peroxidase (GPx) (E-BC-K096, Elabscience Inc.) and Catalase (CAT) (E-BC-K031, Elabscience Biotechnology Inc.) are antioxidant enzymes in human. Enzymatic activity in serum was determined by using ELISA Kits via ELISA reader (BioTek, PowerWave XS2, City, State, USA).

6.6.5 Determination of blood metal elements

One mL of whole blood samples were microwave digested with 3 mL of 65% nitric acid (Ultrapure Reagent, J.T. Baker). Subsequently, we washed the residuals in microwave tubes with 2% nitric acid and then filtered the digested fluids with 0.22 μm filter. The total filtered solutions were stored in 15 mL centrifuge tubes. The levels of Na, Mg, K, Mn, Fe, Ni, Cu, Zn, Sr, As, Se, Mo, Cd, Sb, Ba and Pb were determined by using inductively coupled plasma mass spectrometry (ICP-MS; Agilent 7800).

6.6.6 Analysis of anabolic hormone

Age-dependent muscle breakdown resulted in the decrease of anabolic hormones; therefore, dehydroepiandrosterone sulfate (DHEA-S) was employed in this study. Serum DHEA-S was determined by using ELISA kit (AB108669) via ELISA reader (Bio Tek, PowerWave XS2, City, State, USA).

6.6.7 Analysis of immune function

Human peripheral bloods were separated from whole blood by using PluriMate (#44-91002-11). After separation, Fc receptors in PBMC were blocked by Fc blocker (Biolegend #422302), stained with monoclonal antibodies (CD3-EV450, CD4-AF488, CD8a-AF647, CD25- PE/Cyanine7, (Elabscience Biotechnology Inc.)), and analyzed by the Invitrogen™ Attune™ NxT acoustic focusing cytometer (Thermo Fisher Scientific).

6.7 Questionnaire

6.7.1 Basic information

The researchers with IRB certificate helped subjects to fill their self-reported basic information, including age, disease history, etc. The reporting form was shown in Appendix II.

6.7.2 24-Hour dietary recall

24-Hour dietary recall was to recall past 24 hours food intake and record food items, cooking methods, portion sizes, and calorie calculation. The reporting form was shown in appendix III.

6.7.3 International physical activity questionnaire (iPAQ)

iPAQ was used to estimate the types of intensity of physical activity and sitting time. The reporting form was shown in appendix IV.

6.7.4 Food frequency questionnaire

Food frequency questionnaire was used to estimate long-term dietary conditions. The reporting form was shown in appendix V.

6.7.5 Taiwan lifestyle assessment

Lifestyle questionnaire as a thirteen-item questionnaire which was reported by patients themselves. The reporting format was shown in appendix VI.

6.7.6 Patient Assessment of Constipation symptom (PAC-SYM)

PAC-SYM was used to measure specific symptoms of patients with constipation, was developed in parallel with a complementary instrument to comprehensively measure disease QoL outcomes (PAC-QOL). These instruments could be used separately or together. The reporting format was shown in appendix VII

6.7.7 SARC-F questionnaire

SARC-F was a five-item questionnaire, which was reported by patients themselves to screen for sarcopenia. The reflection was based on the patient's perception of his own strength, ability to walk, stand up from the chair, climb stairs and fall experience. More than 4 points might be at risk of sarcopenia. The reporting format was shown in appendix VIII.

6.7.8 Mini nutrition assessment (MNA)

MNA was used to screen out high-risk groups of malnutrition. The maximum score obtained by MNA was 30 points, between 17-23.5 represents the risk of malnutrition, less than 17 points was malnutrition. The reporting format was shown in appendix IX.

6.8 Statistical analysis

The required sample size was determined using G*Power software, ensuring adequate power to detect meaningful differences. The calculation was based on an estimated effect size of 0.5 (moderate effect), power of 80%, and an alpha level of 0.05, following recommendations for microbiome and clinical intervention studies.

The sample size estimate also accounted for potential dropouts, ensuring robustness in statistical analysis. Randomization was conducted using a computer-generated sequence, stratified by baseline sarcopenia severity to ensure balanced group allocation. Data were presented as median (IQR), number (percentage) or box-and-whisker plots (min-max). Comparisons between two groups were performed with Mann Whitney test or Wilcoxon matched-pairs signed rank test, while comparisons across more than two groups were performed with Kruskal-Wallis test followed by Dunn's post hoc test. Demographic data were analyzed using the Chi-squared test (χ^2 test) or Fisher exact test. A p-value of <0.05 was considered significantly significant.

7. Results

7.1 Sarcopenia vs healthy subjects: Participants evaluation

7.1.1 Basic characteristics

On the basis of sarcopenia criteria, a total of 92 screened participants were eligible for sarcopenia, and a total of 40 screened participants were eligible for non-sarcopenia (Figure 2). Study subjects were enrolled from October 1st 2021 and the last subject completed their 12-weeks treatment on January 13th 2023. 12 subjects were withdrawn during 12-weeks treatment. The PP analysis included all study subjects (n =120) who were randomized to NS, SC, LcS groups. The baseline characteristics of SC and LcS groups were well balanced (Table 3).

The basic information form and lifestyle habits questionnaire enabled us to comprehend the fundamental living conditions of the participants, providing a standard for evaluating subsequent data more effectively.

Results from Table 4 indicated that there were no statistically significant differences in age, gender, education level, annual income, or medical history between the sarcopenia group (SC) and the control group (NS), as indicated by p-values greater than 0.05.

Data from lifestyle on demographic characteristics (Table 5) revealed that the consumption of coffee, tea, and vitamin supplements showed no statistically significant differences between the SC and the NS, with p-values exceeding 0.05. Conversely, alcohol consumption exhibited a statistically significant difference between the SC and the NS, with a higher consumption rate in the NS group, as evidenced by a p-value less than 0.05. In terms of environmental exposure assessments, there were no statistically significant differences between the groups in exposure to kitchen oil fumes, usage frequency of exhaust fans, frequency of incense burning (including sandalwood and other aromatics), smoking frequency, and the probability of exposure to secondhand smoke, all showing p-values greater than 0.05. However, a statistically significant difference was noted in the frequency of mosquito coil use between the SC and the NS, with less usage in the SC group compared to the NS group ($p = 0.028$).

While the statistical results indicated significant differences in alcohol consumption and mosquito coil usage frequency, the majority of participants either did not consume alcohol or use mosquito coils. Consequently, these findings does not support

the conclusion that the sarcopenia group has a lower frequency of alcohol consumption or mosquito coil usage compared to the control group.

7.1.2 Anthropometric measurement

Sarcopenia patients generally exhibited lower muscle mass compared to non-sarcopenic individuals. Consequently, this study employed the InBody S10 to assess intergroup differences in body composition, aiming to confirm the specific body composition disparities between sarcopenic subjects (SC) and control subjects (NS).

Data from Table 6 on body composition revealed that subjects diagnosed with sarcopenia exhibited significantly reduced values in several parameters compared to control subjects. These parameters included waist circumference, body weight, Body Mass Index (BMI), Skeletal Muscle Mass Index (SMI), skeletal muscle mass, soft lean mass, fat-free mass, protein content, fat content, intracellular water, extracellular water, total body water, and whole body phase angle. Collectively, the data indicate that subjects in the sarcopenia group not only meet the clinical criteria for sarcopenia but also exhibit statistically significant differences from the control group in various measured parameters.

7.1.3 Physical performance

The physical performance of elderly individuals was impacted by the natural decline of bodily functions associated with aging, which could lead to chronic diseases. Physical performance tests were essential for rapidly assessing a subject's capabilities in daily living, exercise, and physical activities. This study utilized a battery of tests, including grip strength measurement, chair stand test, walking speed assessment, the TUG test, and the SPPB score, to evaluate the physical performance of the subjects. Additionally, the International Physical Activity Questionnaire (IPAQ) was administered to assess the usual physical activity levels of the subjects.

In the results of the physical performance assessment (Table 7), individuals diagnosed with sarcopenia showed no statistically significant differences in the chair stand test, walking speed, or the TUG test compared to the control group. However, the sarcopenia group demonstrated significantly lower grip strength and SPPB scores relative to controls. Additionally, as outlined in Table 8, there are no statistically significant differences in physical activity levels between the sarcopenia group and

the control group.

7.1.4 Dietary Assessment (MNA, 24h-recall, and Food frequency questionnaire)

This study employed the Mini Nutritional Assessment (MNA), the 24-hour dietary recall method, and the food frequency questionnaire (FFQ) to evaluate the nutritional status of the subjects.

Data from Table 9, utilizing the Mini Nutritional Assessment (MNA), indicated a statistically significant difference between the SC and the NS, with more well-nourished participants in the NS group compared to the SC group ($p = 0.002$).

Results from the 24-hour dietary recall presented in Table 10 showed no statistically significant differences in total caloric intake, carbohydrate, protein, and fat intake percentages between the SC and the NS, all with p-values greater than 0.05.

According to Table 11, which documents dietary intake frequencies, there were no significant differences in the frequencies of fish and deep-sea fish consumption between the SC and the NS, as indicated by p-values greater than 0.05. However, a statistically significant difference waswas observed in the frequency of other seafood consumption between the SC and the NS, with higher consumption the NS group ($p = 0.019$). No significant differences were noted in meat consumption frequencies between the two groups ($p>0.05$). Statistically significant differences were present in the frequencies of consumption of animal organs or blood and processed meats between the SC and the NS, with higher consumption in the SC group, both with p-values less than 0.05. No significant difference was found in the frequency of legume product consumption between the groups ($p>0.05$).

Based on the results, there was no difference in total caloric intake between the SC group and the NS group. However, the SC group might be at risk of malnutrition. The food frequency questionnaire indicate that the SC group has a lower intake frequency of seafood, animal offal or blood, and processed meat products.

7.1.5 Patient Assessment of Constipation symptom

Current research suggested that gastrointestinal symptoms might have systemic effects. The constipation self-assessment scale could evaluate participants' abdominal symptoms (including stomach pain, abdominal cramping, bloating, and discomfort), rectal symptoms (including decreased frequency of bowel movements, straining during defecation, painful defecation, sensation of incomplete evacuation, difficulty

passing stool despite the urge, rectal bleeding or tearing, and rectal burning sensation), and stool hardness. Utilizing this scale could provide a preliminary understanding of whether sarcopenia patients commonly exhibited abnormal gastrointestinal symptoms.

Results from the constipation self-assessment (PAC-SYM) detailed in Table 12 revealed no statistically significant differences in the abdominal symptom scores (encompassing stomach pain, abdominal cramps, abdominal distension, and general abdominal discomfort) between the SC and the NS, with p-values exceeding 0.05. Additionally, the rectal symptom scores, which included variables such as decreased defecation frequency, effortful defecation, painful defecation, sensation of incomplete bowel clearance, urge to defecate without success, rectal bleeding or fissure, and rectal burning, similarly showed no statistically significant differences between the SC and the NS, with all p-values greater than 0.05. No significant differences were observed in stool hardness scores between the two groups ($p>0.05$). Furthermore, the total scores for the non-intervention group (SC) compared to the NS also indicated no significant discrepancies ($p>0.05$).

Based on the above results, there are no difference in gastrointestinal function between sarcopenia patients and non-sarcopenic individuals.

7.1.6 Blood biochemical parameters

Blood biochemical analysis was performed to identify potential differences in biochemical functions between sarcopenia patients and non-sarcopenic individuals, providing a foundation for subsequent experiments.

Analysis of the blood biochemistry data presented in Table 13 indicated that there were no statistically significant alterations in the following biochemical markers for both the sarcopenia group and the control group: Thyroid-Stimulating Hormone (TSH), Free Thyroxine (Free T4), High Sensitivity C-Reactive Protein (hsCRP), Albumin, Pre-albumin, Total Cholesterol (CHOL), High-Density Lipoprotein Cholesterol (HDL-C), Low-Density Lipoprotein Cholesterol (LDL-C), Triglycerides (TG), Hemoglobin A1c (HbA1c), Glucose (GLU AC), Insulin, Homeostatic Model Assessment for Insulin Resistance (HOMA-IR), Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Blood Urea Nitrogen (BUN), Creatinine, Estimated Glomerular Filtration Rate (eGFR), White Blood Cells (WBC), Red Blood Cells (RBC), Hemoglobin (HGB), Hematocrit (HCT), Mean Corpuscular Hemoglobin Concentration (MCHC), Platelets, and Red Cell Distribution Width-Coefficient of

Variation (RDW-CV).

Based on the above results, there are no significant differences in blood biochemical parameters between sarcopenia patients and non-sarcopenic individuals.

7.1.7 T cell subtypes

T cells played an essential role in skeletal muscle repair and fibrosis. Research had shown a positive correlation between higher levels of CD4+ T cells and the prevalence of sarcopenia[45, 46]. In this study, the analysis of T lymphocyte subsets (Table 14) revealed no significant differences in the proportions of CD3+, CD8+, and regulatory T cells between the SC and the NS ($p>0.05$). However, the sarcopenia group exhibited a significantly higher proportion of CD4+ T cells compared to the control group ($p<0.05$). Therefore, the proportion of CD4+ T cells may serve as a potential biomarker for the diagnosis of sarcopenia.

7.1.8 Pro-inflammatory and anti-inflammatory cytokines

Current research predominantly indicated that age-induced inflammatory responses are a primary cause of sarcopenia. Therefore, we measured common inflammatory cytokines, such as IL-6, TNF-α, IL-10, TGF-β, and IL-17, to evaluate their roles in this process.

Table 15 result revealed that individuals in the sarcopenia group exhibited significantly elevated levels of pro-inflammatory cytokines (e.g., IL-6, TNF-α) relative to the control group. Conversely, concentrations of anti-inflammatory cytokines (e.g., IL-10, TGF-β) were significantly reduced in the sarcopenia group. It was noteworthy that the level of the pro-inflammatory cytokine IL-17 was significantly lower in the sarcopenia group compared to the control group.

7.1.9 Antioxidant activity

The activity of antioxidant enzymes in the body decreased with aging, and age-related oxidative stress might contribute to muscle damage. Consequently, this study assessed the activity of antioxidant enzymes in the blood to evaluate whether sarcopenia patients exhibited reduced antioxidant capacity. The data on antioxidant enzyme activity (Table 16) revealed that, except for superoxide dismutase (SOD), which showed no significant difference between the two groups ($p>0.05$), the SC had significantly lower levels of catalase (CAT) and glutathione peroxidase (GPx)

compared to the NS ($p<0.05$). These findings indicate that the sarcopenia group has a generally reduced antioxidant enzyme activity relative to the control group, with significant differences specifically in CAT and GPx levels ($p<0.05$).

7.1.10 Anabolic hormone

Dehydroepiandrosterone sulfate (DHEA-S) has anti-aging effects, but its secretion declines with age. Therefore, by measuring the blood levels of DHEA-S, we could determine whether sarcopenia patients (SC) exhibited a greater tendency towards aging compared to the NS. The data on bone anabolic hormones (Table 17) indicate that DHEA-S levels are lower in the sarcopenia group compared to the control group, although this difference is not statistically significant ($p=0.082$).

7.1.11 Metal concentration

Analysis of the blood metal concentrations detailed in Table 18 reveals significant elevations in nickel (Ni) and antimony (Sb) levels in the sarcopenia group compared to the control group. Conversely, selenium (Se) and lead (Pb) concentrations were significantly lower in the sarcopenia group. No statistically significant differences were observed in the levels of sodium (Na), magnesium (Mg), potassium (K), manganese (Mn), iron (Fe), copper (Cu), zinc (Zn), strontium (Sr), arsenic (As), molybdenum (Mo), cadmium (Cd), and barium (Ba) between the two groups.

7.1.12 Gut microbiota

Alpha diversity analysis based on the Shannon index (Fig. 3A) and Observed features index (Fig. 3B) showed no significant differences between the NS and SC groups ($p > 0.05$). However, β-diversity analysis using Bray–Curtis distance (Fig. 3C) demonstrated partial separation between the two groups (PERMANOVA, $p < 0.05$), indicating differences in overall gut microbial community structure.

The taxonomic composition of the gut microbiota was evaluated at the genus level. Figure 3D shows average relative abundances of the 20 most abundant bacterial genera in both groups, while Figure 3F displays the relative abundances of the top 35 bacterial genera in each subject.

Genus-level comparisons identified distinct bacterial signatures associated with sarcopenia status (Fig 3E, Fig. 4, Table 19). Both LEfSe (Fig 3E) and

MetagenomeSeq analyses (Fig 4) revealed significantly lower abundance of *Bifidobacterium* in SC compared to NS. LEfSe-only findings included increased *Shigella* and *Muribaculum*, and decreased *Catenibacterium* in SC. MetagenomeSeq-only results showed enrichment of *Anaerobutyricum*, *Anaerofilum*, *Gluceribacter*, *Lachnospira*, and *Oscillibacter*, along with depletion of *Duncaniella*, *Subdoligranulum*, and *Tyzzerella* in SC.

Collectively, while microbial richness and evenness were comparable, SC participants exhibited reduced beneficial taxa such as *Bifidobacterium* and *Subdoligranulum*, and increased abundance of genera potentially linked to dysbiosis or inflammation (*Shigella*, *Oscillibacter*, *Anaerobutyricum*), suggesting a sarcopenia-associated gut microbial shift.

7.2 Influence of Fermented Milk Containing Lactobacillus casei Strain Shirota intervention in Sarcopenia elderly

7.2.1 Anthropometric measurement

This study utilized the InBody S10 to evaluate the body composition of participants. The comparison of pre- and post-intervention body measurements in the SC (Table 20) revealed no significant differences in waist circumference, height, weight, BMI, skeletal muscle mass, soft lean mass, fat-free mass, protein, fat, intracellular water, and extracellular water ($p>0.05$). However, significant increases were observed in SMI, total body water, and WBPA ($p<0.05$). In the LcS group, the comparison of pre- and post-intervention body measurements (Table 20) showed no significant differences in waist circumference, height, weight, BMI, SMI, skeletal muscle mass, soft lean mass, fat-free mass, protein, fat, intracellular water, extracellular water, total body water, and WBPA ($p>0.05$). When comparing the post-intervention body measurements between the SC and the LcS group (Table 20), no significant differences were found in waist circumference, height, weight, BMI, SMI, skeletal muscle mass, soft lean mass, fat-free mass, protein, fat, intracellular water, extracellular water, total body water, and WBPA ($p>0.05$). Therefore, the Fermented Milk Containing LcS intervention does not appear to be effective in improving the body composition of sarcopenia patients.

7.2.2 Physical performance

This study utilized a series of assessments to evaluate physical performance, including

grip strength, the chair stand test, walking speed, the Timed Up and Go (TUG) test, and the SPPB score. The International Physical Activity Questionnaire (IPAQ) was also administered to gauge participants' usual physical activity levels. In the SC, pre- and post-test comparisons of physical performance measurements (Table 22) showed no significant differences in grip strength, chair stand test, walking speed, TUG test, and SPPB score ($p>0.05$). Conversely, in the LcS group, significant improvements were observed in grip strength, chair stand test, walking speed, TUG test, and SPPB score after 12 weeks of intervention ($p<0.05$). Further comparison of post-test physical performance between the SC and the LcS group revealed no significant differences in grip strength and the TUG test ($p>0.05$). However, significant improvements were noted in the chair stand test, walking speed, and SPPB score in the LcS group ($p<0.05$). Regarding physical activity levels, no significant differences were found in pre- and post-test comparisons (Table 22) within either the SC or LcS groups, nor in comparisons between the SC and LcS groups ($p>0.05$). These findings suggest that, while the Fermented Milk Containing LcS intervention did not enhance muscle mass, it led to significant improvements in physical performance metrics in sarcopenia patients without affecting their overall physical activity levels.

7.2.3 Dietary Assessment (MNA, 24h-recall)

This investigation utilized the Mini Nutritional Assessment (MNA) and a 24-hour dietary recall to evaluate the nutritional status of participants. According to results from Table 23, the Mini Nutritional Assessment showed no significant changes in nutritional intake between pre- and post-assessment within the sarcopenia non-intervention group (SC) ($p>0.05$). Conversely, significant differences in nutritional intake were noted within the LcS group between the pre- and post-assessments ($p<0.05$). No statistically significant differences were found in post-assessment nutritional status between the sarcopenia non-intervention group (SC) and the LcS group ($p>0.05$).

Data from Table 24, derived from the 24-hour dietary recall, indicated no significant alterations in total calorie consumption, carbohydrate intake percentage, protein intake percentage, or fat consumption during pre- and post-intervention comparisons within the sarcopenia non-intervention group (SC) ($p>0.05$). Similarly, no significant differences were observed within the LcS group in the mentioned dietary metrics between pre- and post-intervention ($p>0.05$). Additionally,

comparative analysis of dietary nutrition status in post-intervention assessments between the two groups also revealed no significant differences ($p>0.05$)."

7.2.4 Constipation symptom assessment

The constipation self-assessment scale was utilized to analyze participants' abdominal symptoms (including stomachache, abdominal cramping, bloating, and discomfort), rectal symptoms (such as decreased defecation frequency, straining, pain during defecation, incomplete evacuation, urgency with difficulty expelling, rectal bleeding or fissures, and burning sensation), and stool hardness. In the sarcopenia non-intervention group (SC), comparisons of pre- and post-intervention assessments (Table 25) of abdominal and rectal symptom scores, stool hardness scores, and overall scores indicated no statistically significant changes associated with constipation ($p>0.05$). In contrast, the LcS group demonstrated significant improvements in abdominal symptom scores and overall scores 12 weeks after Fermented Milk Containing LcS supplementation compared to baseline ($p<0.05$). Nonetheless, there were no significant changes in rectal symptom scores or stool hardness scores post-intervention compared to pre-intervention ($p>0.05$). Subsequent inter-group comparisons post-intervention between the sarcopenia non-intervention group (SC) and the LcS group identified significant improvements in abdominal symptoms after 12 weeks of Fermented Milk Containing LcS intervention ($p<0.05$), whereas rectal symptoms, stool hardness, and overall scores did not exhibit significant differences between the groups ($p>0.05$).

7.2.5 Blood biochemical parameters

To understand whether the blood biochemical profiles of the subjects could impact subsequent experiments and to ensure that the Fermented Milk Containing LcS intervention does not raise health concerns, this study measured blood biochemical parameters before and after the intervention.

In the SC, pre- and post-intervention blood biochemical data (Table 26) revealed no significant changes in TSH, Free T4, hsCRP, albumin, pre-albumin, CHOL, HDL-C, LDL-C, TG, HbA1c, fasting glucose (GLU (AC)), insulin, HOMA-IR, AST, ALT, BUN, creatinine, eGFR, WBC, RBC, HGB, HCT, MCHC, platelet count, and RDW-CV ($p>0.05$).

Similarly, in the LcS group, pre- and post-intervention blood biochemical data

(Table 27) showed no significant changes in TSH, Free T4, hsCRP, albumin, pre-albumin, CHOL, HDL-C, LDL-C, TG, HbA1c, fasting glucose (GLU (AC)), insulin, HOMA-IR, AST, ALT, BUN, creatinine, eGFR, WBC, RBC, HGB, HCT, MCHC, platelet count, and RDW-CV ($p>0.05$).

Comparing the blood biochemical data between the SC and LcS groups (Table 28), there were no significant differences in TSH, hsCRP, albumin, pre-albumin, CHOL, HDL-C, LDL-C, TG, HbA1c, fasting glucose (GLU (AC)), insulin, HOMA-IR, AST, ALT, BUN, creatinine, eGFR, WBC, RBC, HGB, HCT, MCHC, platelet count, and RDW-CV ($p>0.05$). However, in the LcS group, there was a significant reduction in the number of subjects with abnormal Free T4 and fasting glucose (GLU (AC)) values ($p<0.05$), and a significant increased number of subjects with abnormal LDL-C levels ($p<0.05$).

7.2.6 T cell subtypes

The results from Table 14 indicated that the proportion of CD4+ T cells is higher in sarcopenia patients. Therefore, this study aimed to investigate whether the Fermented Milk Containing LcS intervention could adjust the proportion of T cells in the SC group. According to the T lymphocyte data (Table 29), the pre- and post-intervention comparison in the SC group showed no significant differences in the proportions of CD3+, CD4+, CD8+, and regulatory T cells ($p>0.05$). In the LcS group, the pre- and post-intervention comparison also showed no significant differences in the proportions of CD3+, CD8+, and regulatory T cells ($p>0.05$). However, there was significant reduction in the proportion of CD4+ T cells following the Fermented Milk Containing LcS intervention ($p<0.05$. When comparing the SC and LcS groups, no significant differences were observed in the proportions of CD3+, CD4+, CD8+, and regulatory T cells ($p>0.05$). Overall, the Fermented Milk Containing LcS intervention did not result in significant changes in the proportions of T lymphocytes.

7.2.7 Pro-inflammatory and anti-inflammatory cytokines

Cytokines and lower levels of anti-inflammatory cytokines compared to the normal group. Therefore, this study aimed to determine whether the Fermented Milk Containing LcS intervention could alleviate inflammation in the sarcopenia group. According to the data in Table 30, there were no significant differences in IL-6, IL-10, IL-17, and TGF-β in the pre- and post-intervention comparisons within the SC group,

within the LcS group, or between the SC and LcS groups. TNF-α levels showed no significant difference in the pre- and post-intervention comparison within the SC group. However, in the LcS group, the post-intervention TNF-α levels were higher compared to pre-intervention levels, yet significantly lower than those in the SC group. These results suggest that the Fermented Milk Containing LcS intervention can effectively reduce serum TNF-α levels.

7.2.8 Antioxidant activity

According to the data in Table 31, there were no significant differences in the activity of the three antioxidant enzymes in the pre- and post-intervention comparisons within the SC group ($p>0.05$). In the LcS group, there were no significant differences in CAT and SOD activity ($p>0.05$), but GPx activity showed a significant increase after three months of intervention ($p<0.05$). Comparing the SC and LcS groups, there were no significant differences in GPx and SOD activity ($p>0.05$), but CAT activity was significantly lower in the LcS group compared to the SC group ($p<0.05$). These results suggest that while the Fermented Milk Containing LcS intervention may slightly inhibit CAT activity, there were no significant changes in CAT within the LcS group itself. However, the intervention did lead to a significant increase in GPx activity.

7.2.9 Anabolic hormone

The results from Table 17 indicated that DHEA-S levels tend to decrease in sarcopenia patients. Therefore, this study aimed to determine whether the Fermented Milk Containing LcS intervention could increase DHEA-S levels in the body. According to the data in Table 32, there were no significant differences in DHEA-S levels in the pre- and post-intervention comparisons within the SC group, within the LcS group, or between the SC and LcS groups ($p>0.05$). These results suggest that the Fermented Milk Containing LcS intervention does not alter DHEA-S levels in the body.

7.2.10 Metal concentration

Research indicated that some probiotics help remove metal ions from the body. Therefore, this study aimed to determine if Fermented Milk Containing LcS has this capability. According to the data in Table 33, in the SC group, significant changes

were observed in Ni and Ba levels in the blood before and after the test ($p<0.05$). In the LcS group, significant decreases were observed in Na, Mg, K, Mn, Fe, Ni, Cu, Zn, Sr, Sb, Ba, and Pb levels before and after the test ($p<0.05$). When comparing the SC and LcS groups, the LcS group had significantly lower levels of Na, Mg, K, Mn, Fe, Ni, Cu, Zn, Sr, Cd, Sb, Ba, and Pb compared to the SC group ($p<0.05$). The results indicate that the intervention with Fermented Milk Containing LcS helps remove most metal ions from the body.

7.2.11 Gut microbiota composition and microbiota-derived Metabolite Analysis

Gut microbiota analysis revealed compositional differences between the sarcopenia control (SC) group and the LcS intervention group. As shown in Figures 5A and 5B, alpha diversity indices—including the Shannon diversity index and the number of observed features—were slightly higher in the SC group compared to the LcS group; however, these differences did not reach statistical significance, suggesting that LcS intervention had a minimal impact on overall microbial diversity. Beta diversity analysis based on Bray–Curtis dissimilarity (Figure 5C) revealed a partial separation between the SC and LcS groups, indicating subtle differences in microbial community composition that were not statistically distinct.

The taxonomic composition of the gut microbiota was evaluated at the genus level. Figure 5D shows average relative abundances of the 20 most abundant bacterial genera in both groups, while Figure 5E displays the relative abundances of the top 35 bacterial genera in each subject.

Differential abundant genera between the groups were identified using LEfSe (Figure 5F) and MegagenomeSeq (Figure 6). Notably, *Bifidobacterium*, *Lacrimispora*, and *Lacticaseibacillus* were consistently higher, while *Cloacibacillus*, *Gehongia*, *Muribaculum*, *Negativibacillus*, and *Oscillibacter* were consistently lower across both methods (Table 34).

In addition to microbial composition, microbial metabolites were profiled using LC-MS/MS, including short-chain fatty acids (SCFAs), indole derivatives such as indole-3-propionic acid (IPA), and trimethylamine-N-oxide (TMAO). The results are presented in Table 35. Although acetic acid levels were significantly lower in the SC group compared to the NS group, no change was observed compared to the LcS group. Notably, isovaleric acid levels were significantly higher in the SC group compared to both the NS and LcS groups, suggesting that Fermented Milk Containing LcS

intervention may mitigate the elevation of isovaleric acid associated with sarcopenia.

7.3 Spearman correlations between gut microbiota and specific sarcopenia parameter

Based on the relative abundance in SC and LcS subjects, the top 25 bacterial species were selected for analysis. Spearman correlation heat maps were constructed to examine associations between gut microbiota and various parameters, including physical examinations, body composition, cytokines, DHEA-S, CD4+ T cell count, biochemical parameters, heavy metals, and metabolites (Figures 7-12).

The heat map in Figure 7 presents the correlations between gut microbiota and physical performance metrics. Notably, *Lacrimispora* exhibited a negative correlation with the Timed Up and Go (TUG) test, while *Bifidobacterium* showed positive correlations with the Short Physical Performance Battery (SPPB) score and walking speed. Additionally, *Klebsiella* was positively correlated with the chair stand test. These findings suggest that *Bifidobacterium*, *Klebsiella*, and *Lacrimispora* may influence physical function in SC and LcS subjects.

Figure 8 displays the correlations between gut microbiota and body composition metrics. *Muribaculum* was positively associated with fat-free mass, protein, and extracellular water. In contrast, *Parabacteroides* showed a negative association with body weight. *Veillonella* exhibited negative correlations with multiple parameters, including height, weight, skeletal muscle mass, soft lean mass, fat-free mass, protein, total body water, intracellular water, and extracellular water. Similarly, *Lacticaseibacillus* was negatively correlated with body weight, skeletal muscle mass index (SMI), skeletal muscle mass, fat-free mass, total body water, intracellular water, and whole-body phase angle. Conversely, *Negativibacillus* showed positive correlations with height, skeletal muscle mass, soft lean mass, fat-free mass, protein, total body water, intracellular water, and extracellular water. These results indicate that specific bacterial species may play roles in regulating body composition.

Figure 9 highlights correlations between gut microbiota and immune-related parameters. *Odoribacter* was negatively associated with IL-10, whereas *Veillonella* showed a positive correlation with IL-10. Additionally, *Lacticaseibacillus* exhibited a negative correlation with TNF-α. These results suggest that variations in gut microbiota composition may influence cytokine regulation and immune responses.

Figure 10 presents correlations between gut microbiota and biochemical

parameters. *Odoribacter* was positively associated with pre-albumin but negatively correlated with free-T4, high-sensitivity C-reactive protein (HS-CRP), and glutamic oxaloacetic transaminase (GOT). *Parabacteroides* was negatively associated with pre-albumin. *Klebsiella* exhibited positive correlations with thyroid-stimulating hormone (TSH) and blood urea nitrogen (BUN) but negative correlations with cholesterol (CHOL), low-density lipoprotein cholesterol (LDL-C), and insulin. *Bifidobacterium* was positively correlated with CHOL and high-density lipoprotein cholesterol (HDL-C). *Veillonella* showed positive associations with free-T4 and HDL-C but a negative association with creatinine. Similarly, *Lacticaseibacillus* was positively correlated with free-T4 but negatively correlated with pre-albumin, creatinine, white blood cell count (WBC), and hematocrit (HCT). *Negativibacillus* was positively associated with BUN and WBC but negatively associated with free-T4, GOT, and mean corpuscular hemoglobin concentration (MCHC). *Oscillibacter* was positively associated with red blood cell count (RBC) but negatively correlated with free-T4. *Gehongia* was positively associated with BUN but negatively correlated with CHOL. *Lacrimispora* was positively associated with glutamic pyruvic transaminase (GPT) but negatively correlated with WBC and HCT. These findings suggest that specific gut microbiota species may play roles in lipid metabolism, insulin sensitivity, and hematological regulation.

Figure 11 illustrates correlations between gut microbiota and heavy metal concentrations. *Odoribacter* showed a negative correlation with nickel (Ni), while *Parabacteroides* was negatively associated with strontium (Sr). *Klebsiella* exhibited positive correlations with Zinc (Zn), selenium (Se), and lead (Pb). *Megasphaera* was positively associated with sodium (Na), antimony (Sb), and barium (Ba). In contrast, *Lacticaseibacillus* displayed negative correlations with multiple heavy metals, including Na, magnesium (Mg), potassium (K), iron (Fe), Ni, Sr, cadmium (Cd), Sb, Ba, and Pb. *Negativibacillus* was positively associated with Na and K, while *Oscillibacter* showed a negative correlation with Cd. *Gehongia* exhibited a positive correlation with Na. These results indicate that gut microbiota composition may influence heavy metal regulation.

Figure 12 presents correlations between gut microbiota and metabolite levels. *Muribaculum* was positively associated with isobutyric acid and valeric acid, while *Parabacteroides* showed a negative correlation with butyric acid. *Klebsiella* was positively correlated with isobutyric acid. *Veillonella* displayed positive associations

with acetic acid, propionic acid, and indolepropionic acid (IPA) but a negative correlation with isovaleric acid. *Lacticaseibacillus* was negatively associated with isovaleric acid. Conversely, *Negativibacillus* was positively correlated with isovaleric acid but negatively associated with acetic acid. *Oscillibacter* was positively associated with isovaleric acid but negatively correlated with acetic acid, propionic acid, and IPA. *Gehongia* was positively associated with isovaleric acid but negatively correlated with propionic acid. These findings suggest that the Fermented Milk Containing LcS intervention may significantly influence microbial-metabolite interactions.

8. Discussion

This study investigated the potential effects of fermented milk containing Lactobacillus casei Shirota (LcS) on sarcopenia-related biomarkers, inflammatory cytokines, trace metals, and physical performance. While previous research has demonstrated the probiotic benefits of LcS on gastrointestinal and immune function, its potential role in skeletal muscle health and systemic metal modulation remains underexplored. By comparing sarcopenia (SC), non-sarcopenia (NS), and LcS-intervened groups over a 12-week period, this study provides new insights into the multifaceted impacts of LcS supplementation in aging populations.

Overview and Primary Endpoint

This study investigated whether a 12-week intervention with Fermented Milk Containing *Lactobacillus casei* strain Shirota (LcS) could beneficially remodel gut microbiota and improve clinical parameters associated with sarcopenia in elderly individuals. The primary endpoint focused on microbial composition, while secondary endpoints included microbial metabolites, physical performance, antioxidant and inflammatory biomarkers, immune modulation, and heavy metal modulation.

Although α- and β-diversity indices showed no statistically significant changes, genus- and species-level analyses revealed distinct compositional shifts. Both LEfSe and MetagenomeSeq analyses demonstrated enrichment of health-promoting genera—including *Bifidobacterium*, *Lacticaseibacillus*, and *Lacrimispora*—accompanied by a reduction in pro-inflammatory genera such as *Muribaculum* and *Oscillibacter*. These results indicate that LcS supplementation helps shift the gut microbial community toward a more homeostatic and anti-inflammatory state, consistent with literature describing the role of probiotics in correcting age-related dysbiosis.

Increases in *Bifidobacterium* and *Lacticaseibacillus* (formerly part of the

Lactobacillus genus) are of particular interest. These genera have been associated with improved muscle mass and strength. For instance, a systematic review reported that higher levels of *Bifidobacterium* and *Lactobacillus* species were linked to increased skeletal muscle mass and function in both animal and human models of sarcopenia [49].

The observed increase in *Lacrimispora* abundance may also carry functional relevance. A recent study in mice demonstrated that certain *Lacrimispora* species improved locomotor function, possibly via modulation of host-microbiota signaling axes [50]. Conversely, *Muribaculum* has been negatively associated with physical performance and lean mass, appearing more frequently in frail individuals. This negative association was noted in the aforementioned systematic review, and further supported by recent findings linking *Muribaculum*, *Negativibacillus*, and *Oscillibacter* to frailty indicators in older adults [51]. The decreased abundance of these taxa in the LcS group may reflect a microbial shift away from profiles linked to sarcopenic risk.

Currently, limited evidence exists for *Cloacibacillus* and *Gehongia* in relation to muscle physiology or sarcopenia. These genera remain poorly characterized. However, *Cloacibacillus* has been associated with mucosal inflammation and epithelial barrier disruption in some clinical contexts, which may indirectly relate to chronic low-grade inflammation implicated in muscle catabolism. No studies were found connecting *Gehongia* to sarcopenia or musculoskeletal outcomes, underscoring the need for further research into these rare genera.

Microbiota-Derived Metabolites: Functional Implications

Microbial metabolite analysis corroborated the compositional findings. While most short-chain fatty acids (SCFAs) remained stable, acetic acid—commonly reduced in sarcopenic individuals—showed an increasing trend following Fermented Milk Containing LcS supplementation. Notably, isovaleric acid, associated with proteolytic fermentation and muscle degradation, significantly decreased. These findings suggest that Fermented Milk Containing LcS supports a functional shift in microbial metabolism, favoring saccharolytic pathways associated with colonic and systemic health.

Secondary Endpoints

In addition to changes in gut microbiota, this study assessed a broad range of secondary endpoints, including physical performance, muscle function, heavy metal, inflammatory cytokines, antioxidant enzyme activity, anabolic hormones, T lymphocyte subsets, and serum biochemical parameters. These measures were chosen to elucidate potential systemic effects of Fermented Milk Containing LcS beyond the gastrointestinal tract and explore mechanisms underlying sarcopenia progression and its mitigation.

Physical Performance and Muscle Function

Despite the absence of significant changes in muscle mass, the LcS group exhibited improved physical performance across multiple domains: chair stand time, walk speed, and SPPB score. These results suggest that Fermented Milk Containing LcS may promote neuromuscular efficiency or reduce systemic inflammation, rather than stimulating direct hypertrophic effects. These outcomes are clinically meaningful, as enhanced physical function can significantly reduce fall risk and improve quality of life in the elderly.

Heavy Metal Modulation: A Novel Probiotic Mechanism

A particularly novel and impactful finding was the significant reduction in multiple trace and heavy metals—including Ni, Sb, Ba, and Pb—exclusively in the LcS group. Additional decreases were noted for Na, Mg, K, Mn, Fe, Cu, Zn, Sr, and Cd. These reductions suggest that Fermented Milk Containing *Lactobacillus casei* Shirota (LcS), either directly or via microbiota restructuring, facilitates metal ion sequestration, transformation, or fecal elimination [52].

Given that chronic exposure to heavy metals contributes to oxidative stress, mitochondrial damage, and neuromuscular dysfunction, Fermented Milk Containing LcS may offer a unique probiotic-based avenue for environmental toxin mitigation in aging populations. Previous studies have indicated that *Lactobacillus casei* strains can bind and reduce gastrointestinal absorption of non-essential or toxic metals such as cadmium, lead, and arsenic through mechanisms like chelation and surface adsorption.

It is worth noting, however, that in addition to toxic metals, reductions in essential elements such as magnesium (Mg) and zinc (Zn) were also observed. From a nutritional and clinical standpoint, both Mg and Zn are vital for muscle synthesis,

antioxidant defense, immune modulation, and neuromuscular function [53, 54]. Their decline might raise concerns about nutrient deficiency or impaired absorption. However, several contextual factors support a more nuanced interpretation. First, the post-intervention concentrations of Mg and Zn in the LcS group remained within clinically accepted normal reference ranges [55], and there was no evidence of malnutrition based on MNA scores, dietary intake, or 24-hour recall data.

Second, rather than indicating deficiency, the observed decreases in circulating Zn and Mg may reflect redistribution or enhanced cellular uptake—especially in metabolically active tissues like muscle—potentially mediated by improved gut barrier integrity or systemic inflammation reduction [56]. This interpretation is supported by the concurrent improvements in physical performance markers such as walking speed, chair stand time, and SPPB scores observed in the LcS group. In this context, lower serum levels of Zn and Mg may indicate more efficient utilization, not loss.

Third, alterations in gut microbiota composition following LcS supplementation may also influence micronutrient absorption and retention kinetics[57], though no significant differences were observed in overall micronutrient intake across study groups. These hypotheses warrant further investigation through future studies incorporating intracellular metal analysis, metalloprotein profiling, and longer follow-up to assess long-term micronutrient status and safety. Although our study did not show significant changes in skeletal muscle mass, the LcS group demonstrated measurable improvements in functional outcomes. These include better chair stand performance, increased gait speed, and higher SPPB scores—parameters closely linked to fall risk and frailty in older adults [58-60]. Recent literature suggests that probiotics may influence neuromuscular function not only by improving nutrient status and microbiota composition but also by lowering systemic inflammation and oxidative stress—key contributors to sarcopenia and motor decline [61, 62].

Additionally, the reduction in neurotoxic metals (e.g., Pb, Cd) may contribute to improved neuromuscular coordination and cognitive-motor integration [63]. Therefore, even in the absence of measurable muscle mass gains, the overall functional improvements seen in the LcS group point to a clinically meaningful benefit of LcS as a multidimensional strategy to support older adults. Through anti-inflammatory, antioxidative, microbiota-modulating, and potential heavy metal modulation mechanisms, LcS supplementation may help preserve physical

independence, reduce fall risk, and enhance quality of life.

Inflammatory Cytokines

The LcS group experienced a significant reduction in TNF-α, a key pro-inflammatory cytokine associated with muscle catabolism and chronic inflammation. Other cytokines (IL-6, IL-10, IL-17, and TGF-β) did not show significant changes. This TNF-α decline highlights the role of gut-immune interactions in mediating systemic inflammatory responses and suggests that Fermented Milk Containing LcS exerts targeted anti-inflammatory effects via microbiota modulation.

Interestingly, although most previous studies reported an increase in IL-17 in sarcopenic or pro-inflammatory conditions, our data demonstrated a lower IL-17 level in the sarcopenic group (SC) compared to the non-sarcopenic group (NS). This finding may be attributed to several possible explanations.

First, it is known that IL-17 levels are influenced by metabolic conditions such as insulin resistance and components of metabolic syndrome. Given that our inclusion/exclusion criteria did not exclude participants with metabolic comorbidities, it is plausible that the NS group may have included individuals with elevated IL-17 levels related to these underlying conditions. This would result in a relatively higher IL-17 concentration in the NS group.

Second, because the NS group participants were recruited via the same sarcopenia screening pipeline, they may not represent a typical “healthy” reference group. The proximity in their health status to the SC group may have minimized the inflammatory contrast commonly seen in other case-control designs.

These factors should be considered when interpreting the unexpected IL-17 profile observed in our cohort, and future studies may benefit from including a metabolically healthy control group to clarify this observation.

Antioxidant Enzyme Activity

Among the antioxidant enzymes measured, only catalase (CAT) activity showed a significant difference between groups, with lower CAT activity observed in the LcS group compared to the SC group. This between-group difference may reflect a reduced oxidative burden, potentially mediated by changes in microbiota composition or reduced exposure to pro-oxidant stimuli such as heavy metals. Other enzymes,

including glutathione peroxidase (GPx), did not show significant intergroup differences and are therefore not further interpreted in this context.

Immune Modulation

Flow cytometry revealed a trend toward reduced CD4+ T cell proportions within the LcS group ($p < 0.05$). Although not statistically significant and with no difference observed between groups, this trend aligns with prior observations that elevated CD4+ T cells are linked to sarcopenia. Regulatory T cells (Tregs) remained stable; however, their activity could be involved in maintaining mucosal tolerance and systemic immune balance. Overall, these changes support the potential immunomodulatory role of Fermented Milk Containing LcS through gut-mediated signaling.

Anabolic Hormones and Nutritional Indices

Serum levels of DHEA-S, a hormone associated with muscle integrity and aging, remained unchanged, suggesting that Fermented Milk Containing LcS-driven improvements in function are unlikely to be hormone-mediated. Nutritional status assessed by the Mini Nutritional Assessment (MNA) improved post-intervention, indicating enhanced nutritional resilience. Notably, gastrointestinal symptoms related to constipation significantly improved in the LcS group, which may be partially attributable to microbiota restructuring; however, this effect is unlikely to be mediated by a global increase in SCFA production, as only selected metabolites showed significant changes (e.g., decreased isovaleric acid).

Although the total daily caloric intake in both groups appears lower than WHO general recommendations (2,000–3,000 kcal for men and 1,600–2,400 kcal for women), this finding is consistent with national data from the Taiwanese older adult population. According to the Nutrition and Health Survey in Taiwan (NAHSIT 2013–2016), the average energy intake for men and women aged 65 and above is 1,817 kcal and 1,399 kcal, respectively, which closely aligns with our results.

This trend may be attributed to multiple factors including age-related reductions in basal metabolic rate and appetite, cultural dietary patterns emphasizing plant-based and low-fat meals, and the relatively low physical activity level of participants as shown in IPAQ assessments. Notably, most participants were women, further lowering the overall caloric average.

Importantly, no participant met the criteria for clinical malnutrition as assessed

by the Mini Nutritional Assessment (MNA) or 24-hour dietary recalls. Therefore, the lower energy intake observed should be interpreted as physiologically and culturally normative rather than indicative of nutritional insufficiency.

Serum Biochemical Safety Profile

Comprehensive serum biochemical profiles—including lipid panels, liver and kidney function, glucose metabolism, and hematologic markers—remained stable and within reference ranges post-intervention. These data confirm that Fermented Milk Containing LcS supplementation is safe and well-tolerated in elderly individuals with sarcopenia.

Integrated Mechanistic Interpretation

The integrated findings from microbial, metabolic, immune, and functional endpoints suggest that Fermented Milk Containing LcS acts through multi-layered biological mechanisms, including:

- Microbiota remodeling toward eubiotic, anti-inflammatory profiles
- Metabolite modulation promoting beneficial SCFAs and reducing toxic fermentation products
- Improved physical function independent of muscle hypertrophy.
- Enhancing heavy metal modulation, potentially indicating a reduction in oxidative stress burden, as supported by deceased CAT activity.
- Reduction in TNF-α, indicating systemic anti-inflammatory effects

This constellation of effects points to Fermented Milk Containing LcS as a metabolic and immunological modulator with potential to reduce sarcopenia risk and promote healthy aging.

Translational Implications and Future Directions

These results position Fermented Milk Containing LcS as a promising low-risk, microbiota-targeted intervention with broad applicability in geriatric care. Beyond sarcopenia, the observed heavy metal modulation and anti-inflammatory effects may benefit individuals exposed to environmental toxins or those at risk of metabolic and degenerative disorders.

Future studies should explore:

- Functional metagenomics to identify microbial genes responsible for metal metabolism
- Mechanistic studies on the gut-muscle axis
- Long-term trials assessing durability of benefits and impact on clinical outcomes such as fall risk and hospitalization

Limitations

One limitation of this study is its open-label design, which may introduce various biases, particularly in subjective outcomes; to address this, we contextualized our findings by evaluating objective measures, including serum biomarkers and gut microbiota. Additionally, the NS group may have been approaching frailty thresholds, despite recruitment of non-sarcopenic older adults based on the Asian Working Group for Sarcopenia (AWGS) criteria, which focus primarily on muscle-related indicators. This is suggested by the median usual walking speed in the NS group, which fell below 1.0 m/s, as well as by comparisons with broader sarcopenia criteria proposed by Fried et al. and the Japanese CHS adaptation [47,48]. These observations underscore the complexity of functional decline in aging and highlight the need for multi-dimensional screening strategies that extend beyond muscle mass alone in future studies.

Conclusion

Fermented Milk Containing LcS supplementation over 12 weeks in elderly individuals with sarcopenia led to:

- Remodeling of gut microbiota composition
- Functional metabolic improvements
- Enhanced physical performance
- Reduction of pro-inflammatory markers
- Modulation of environmental heavy metals
- Reduced oxidative stress burden

Together, these findings support the therapeutic potential of Fermented Milk Containing LcS as a multi-targeted strategy for sarcopenia management and healthy aging.

References

1. Morley, J.E., S.D. Anker, and S. von Haehling, *Prevalence, incidence, and clinical impact of sarcopenia: facts, numbers, and epidemiology-update 2014.* J Cachexia Sarcopenia Muscle, 2014. **5**(4): p. 253-9.
2. Bianchi, L., et al., *Prevalence and Clinical Correlates of Sarcopenia, Identified According to the EWGSOP Definition and Diagnostic Algorithm, in Hospitalized Older People: The GLISTEN Study.* J Gerontol A Biol Sci Med Sci, 2017. **72**(11): p. 1575-1581.
3. Marzetti, E., et al., *Sarcopenia: an overview.* Aging Clin Exp Res, 2017. **29**(1): p. 11-17.
4. Newman, A.B., et al., *Strength and muscle quality in a well-functioning cohort of older adults: the Health, Aging and Body Composition Study.* J Am Geriatr Soc, 2003. **51**(3): p. 323-30.
5. Visser, M., et al., *Relationship of interleukin-6 and tumor necrosis factor-alpha with muscle mass and muscle strength in elderly men and women: the Health ABC Study.* J Gerontol A Biol Sci Med Sci, 2002. **57**(5): p. M326-32.
6. Miller, M.S., et al., *Age-related slowing of myosin actin cross-bridge kinetics is sex specific and predicts decrements in whole skeletal muscle performance in humans.* J Appl Physiol (1985), 2013. **115**(7): p. 1004-14.
7. Miller, M.S., D.M. Callahan, and M.J. Toth, *Skeletal muscle myofilament adaptations to aging, disease, and disuse and their effects on whole muscle performance in older adult humans.* Front Physiol, 2014. **5**: p. 369.
8. Bian, A.L., et al., *A study on relationship between elderly sarcopenia and inflammatory factors IL-6 and TNF-alpha.* Eur J Med Res, 2017. **22**(1): p. 25.
9. Harman, D., *Aging: a theory based on free radical and radiation chemistry.* J Gerontol, 1956. **11**(3): p. 298-300.
10. Nacarelli, T., A. Azar, and C. Sell, *Aberrant mTOR activation in senescence and aging: A mitochondrial stress response?* Exp Gerontol, 2015. **68**: p. 66-70.
11. Deane, C.S., et al., *"Nutraceuticals" in relation to human skeletal muscle and exercise.* Am J Physiol Endocrinol Metab, 2017. **312**(4): p. E282-E299.
12. Gomez-Cabrera, M.C., et al., *Oral administration of vitamin C decreases muscle mitochondrial biogenesis and hampers training-induced adaptations in endurance performance.* Am J Clin Nutr, 2008. **87**(1): p. 142-9.
13. Glover, E.I., et al., *Little change in markers of protein breakdown and oxidative stress in humans in immobilization-induced skeletal muscle atrophy.* Appl Physiol Nutr Metab, 2010. **35**(2): p. 125-33.
14. Montiel-Castro, A.J., et al., *The microbiota-gut-brain axis: neurobehavioral correlates, health and sociality.* Front Integr Neurosci, 2013. **7**: p. 70.
15. Cho, I. and M.J. Blaser, *The human microbiome: at the interface of health and disease.* Nat Rev Genet, 2012. **13**(4): p. 260-70.
16. Heintz, C. and W. Mair, *You are what you host: microbiome modulation of the aging process.* Cell, 2014. **156**(3): p. 408-11.
17. Claesson, M.J., et al., *Gut microbiota composition correlates with diet and health in the elderly.* Nature, 2012. **488**(7410): p. 178-84.
18. Yatsunenko, T., et al., *Human gut microbiome viewed across age and geography.* Nature, 2012. **486**(7402): p. 222-7.
19. Sharon, G., et al., *The Central Nervous System and the Gut Microbiome.* Cell, 2016. **167**(4): p. 915-932.

20. Langille, M.G., et al., *Microbial shifts in the aging mouse gut.* Microbiome, 2014. **2**(1): p. 50.
21. Magrone, T. and E. Jirillo, *The interaction between gut microbiota and age-related changes in immune function and inflammation.* Immun Ageing, 2013. **10**(1): p. 31.
22. Thevaranjan, N., et al., *Age-Associated Microbial Dysbiosis Promotes Intestinal Permeability, Systemic Inflammation, and Macrophage Dysfunction.* Cell Host Microbe, 2017. **21**(4): p. 455-466 e4.
23. Cevenini, E., D. Monti, and C. Franceschi, *Inflamm-ageing.* Curr Opin Clin Nutr Metab Care, 2013. **16**(1): p. 14-20.
24. Franceschi, C., et al., *Immunosenescence in humans: deterioration or remodelling?* Int Rev Immunol, 1995. **12**(1): p. 57-74.
25. Franceschi, C., et al., *Inflammaging and anti-inflammaging: a systemic perspective on aging and longevity emerged from studies in humans.* Mech Ageing Dev, 2007. **128**(1): p. 92-105.
26. O'Mahony, S.M., et al., *Serotonin, tryptophan metabolism and the brain-gut-microbiome axis.* Behav Brain Res, 2015. **277**: p. 32-48.
27. Yano, J.M., et al., *Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis.* Cell, 2015. **161**(2): p. 264-76.
28. Simpson, H.L. and B.J. Campbell, *Review article: dietary fibre-microbiota interactions.* Aliment Pharmacol Ther, 2015. **42**(2): p. 158-79.
29. Conterno, L., et al., *Obesity and the gut microbiota: does up-regulating colonic fermentation protect against obesity and metabolic disease?* Genes Nutr, 2011. **6**(3): p. 241-60.
30. Rios-Covian, D., et al., *Intestinal Short Chain Fatty Acids and their Link with Diet and Human Health.* Front Microbiol, 2016. **7**: p. 185.
31. Pike, J. and R.K. Chandra, *Effect of vitamin and trace element supplementation on immune indices in healthy elderly.* Int J Vitam Nutr Res, 1995. **65**(2): p. 117-21.
32. Nogawa, K. and T. Kido, *Biological monitoring of cadmium exposure in itai-itai disease epidemiology.* Int Arch Occup Environ Health, 1993. **65**(1 Suppl): p. S43-6.
33. Harada, M., *Minamata disease: methylmercury poisoning in Japan caused by environmental pollution.* Crit Rev Toxicol, 1995. **25**(1): p. 1-24.
34. Vig, E.K. and H. Hu, *Lead toxicity in older adults.* J Am Geriatr Soc, 2000. **48**(11): p. 1501-6.
35. Yoo, J.I., et al., *High Levels of Heavy Metals Increase the Prevalence of Sarcopenia in the Elderly Population.* J Bone Metab, 2016. **23**(2): p. 101-9.
36. Hanna, B.S., et al., *The gut microbiota promotes distal tissue regeneration via RORgamma(+) regulatory T cell emissaries.* Immunity, 2023. **56**(4): p. 829-846 e8.
37. Nelke, C., et al., *Skeletal muscle as potential central link between sarcopenia and immune senescence.* EBioMedicine, 2019. **49**: p. 381-388.
38. Miyazaki, K. and T. Matsuzaki, *Health properties of milk fermented with Lactobacillus casei strain shirota (LcS).* 2008. p. 165-208.
39. Benton, D., C. Williams, and A. Brown, *Impact of consuming a milk drink containing a probiotic on mood and cognition.* Eur J Clin Nutr, 2007. **61**(3): p. 355-61.
40. Rao, A.V., et al., *A randomized, double-blind, placebo-controlled pilot study of a probiotic in emotional symptoms of chronic fatigue syndrome.* Gut Pathog,

2009. **1**(1): p. 6.
41. Kato-Kataoka, A., et al., *Fermented milk containing Lactobacillus casei strain Shirota prevents the onset of physical symptoms in medical students under academic examination stress.* Benef Microbes, 2016. **7**(2): p. 153-6.
42. Chen, L.H., et al., *Probiotic supplementation attenuates age-related sarcopenia via the gut-muscle axis in SAMP8 mice.* J Cachexia Sarcopenia Muscle, 2022. **13**(1): p. 515-531.
43. Wang, Y.F., et al., *Endothelium-Derived 5-Methoxytryptophan Is a Circulating Anti-Inflammatory Molecule That Blocks Systemic Inflammation.* Circ Res, 2016. **119**(2): p. 222-36.
44. Wu, J.Y., et al., *Cancer-Derived Succinate Promotes Macrophage Polarization and Cancer Metastasis via Succinate Receptor.* Mol Cell, 2020. **77**(2): p. 213-227 e5.
45. Dumke, B.R. and S.J. Lees, *Age-related impairment of T cell-induced skeletal muscle precursor cell function.* Am J Physiol Cell Physiol, 2011. **300**(6): p. C1226-33.
46. Nascimento, L.C.P.d., et al., *Sarcopenia and consumptive syndrome in HIV-infected patients receiving antiretroviral therapy in a public hospital in Northeast Brazil.* Revista chilena de nutrición, 2020. **47**(3): p. 430-442.
47. Fried, L.P., et al., *Frailty in older adults: evidence for a phenotype.* J Gerontol A Biol Sci Med Sci, 2001. **56**(3): p. M146-56.
48. Komiya, H., et al., *Validation of the Japanese version of the Clinical Frailty Scale.* Geriatr Gerontol Int, 2025. **25**(3): p. 411-417.
49. Liu, C., et al., *Understanding the gut microbiota and sarcopenia: a systematic review.* J Cachexia Sarcopenia Muscle, 2021. **12**(6): p. 1393-1407.
50. Ahn, J.S., et al., *Identification of the Intestinal Microbes Associated with Locomotion.* Int J Mol Sci, 2023. **24**(14).
51. Le Cosquer, G., et al., *Pathogenicity of commensal gut biofilm in prefrail aging.* NPJ Biofilms Microbiomes, 2025. **11**(1): p. 84.
52. Krajewski, K., *Heavy metals, noradrenaline/adrenaline ratio, and microbiome-associated hormone precursor metabolites: biomarkers for social behaviour, ADHD symptoms, and executive function in children.* Sci Rep, 2025. **15**(1): p. 19006.
53. Cruz, K.J., A.R. de Oliveira, and N. Marreiro Ddo, *Antioxidant role of zinc in diabetes mellitus.* World J Diabetes, 2015. **6**(2): p. 333-7.
54. Grober, U., J. Schmidt, and K. Kisters, *Magnesium in Prevention and Therapy.* Nutrients, 2015. **7**(9): p. 8199-226.
55. *Dietary Reference Intakes for Calcium, Phosphorus, Magnesium, Vitamin D, and Fluoride*, in *Dietary Reference Intakes for Calcium, Phosphorus, Magnesium, Vitamin D, and Fluoride*. 1997: Washington (DC).
56. Ferenc, K., et al., *Modulation of the Gut Microbiota by Nutrition and Its Relationship to Epigenetics.* Int J Mol Sci, 2024. **25**(2).
57. Zhai, Q., A. Narbad, and W. Chen, *Dietary strategies for the treatment of cadmium and lead toxicity.* Nutrients, 2015. **7**(1): p. 552-71.
58. Rolland, Y., et al., *Physical performance measures as predictors of mortality in a cohort of community-dwelling older French women.* Eur J Epidemiol, 2006. **21**(2): p. 113-22.
59. Studenski, S., et al., *Gait speed and survival in older adults.* JAMA, 2011. **305**(1): p. 50-8.
60. Beck Jepsen, D., et al., *Predicting falls in older adults: an umbrella review of*

instruments assessing gait, balance, and functional mobility. BMC Geriatr, 2022. **22**(1): p. 615.
61. Ticinesi, A., et al., *Gut Microbiota, Muscle Mass and Function in Aging: A Focus on Physical Frailty and Sarcopenia*. Nutrients, 2019. **11**(7).
62. Suvorov, A., *Gut microbiota, probiotics, and human health.* Biosci Microbiota Food Health, 2013. **32**(3): p. 81-91.
63. Sanders, A.P., B. Claus Henn, and R.O. Wright, *Perinatal and Childhood Exposure to Cadmium, Manganese, and Metal Mixtures and Effects on Cognition and Behavior: A Review of Recent Literature.* Curr Environ Health Rep, 2015. **2**(3): p. 284-94.

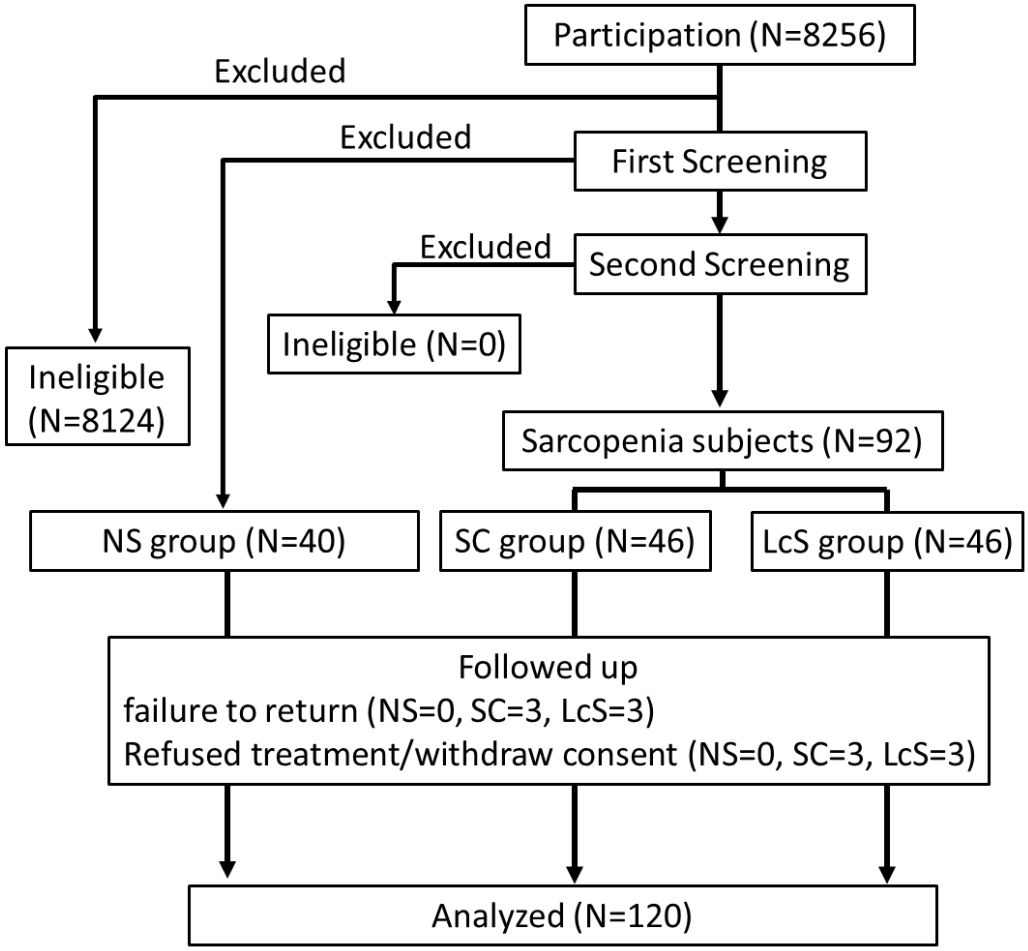


Figure 2. Participant flowchart of patients eligible, recruited, numbers followed up and included in analysis.

Table 3. Baseline characteristics for the PP population

Characteristic	NS (N=40) [a]	SC (N=40) [a]	LcS (N=40) [a]
Age (year)	72.56±4.33	73.88±5.66	73.77±5.70
Gender (Male/Female)	12/28	10/30	5/35
Height (cm)	156.91±7.37	154.84±6.68	153.46±6.98
Weight (kg)	60.56±8.35	49.61±5.81	48.86±6.40
BMI (kg/m^2)	24.57±2.65	20.75±2.49	20.73±2.28
SMI (kg/m2)	6.70±1.00	5.49±0.65	5.28±0.58
Skeletal Muscle Mass (kg)	22.47±4.80	18.17±2.60	17.42±2.64
Fat (kg)	18.93±6.07	14.95±4.29	15.65±4.42
Total Body Water (kg)	30.59±6.32	25.33±3.34	24.40±3.26
SARC-F (score)	1.00±1.36	1.00±1.23	1.00±1.79
Calf circumference (cm)	35.25±3.06	32.00±2.27	31.50±2.07
Handgrip (kg)	22.17±7.46	17.77±5.70	17.83±5.49
Chair stand test (second)	12.91±5.05	14.39±10.59	13.90±4.63
Walk speed (m/s)	0.89±0.21	0.83±0.36	0.86±0.30
SPPB score (pt)	11.00±1.82	10.00±2.04	10.00±2.07
TUG test (second)	10.12±3.18	11.05±5.11	11.64±3.02
Albumin (g/dL)	4.45±0.30	4.40±0.23	4.50±0.41
GLU(AC) (mg/dL)	98.00±22.10	99.50±23.79	93.00±22.94

[a] Data were presented as mean±SD

Sarcopenia vs healthy subjects:

Table 4. Descriptive statistics of basic information on demographics of subjects.

Variable	NS (n=40)	SC (n=80)	*p*-value
Age, median (IQR)	73 (5.00)	74 (8.75)	0.408 [a]
Gender, n (%)			
Female	28 (70.0)	65 (81.25)	0.164 [b]
Male	12 (30.0)	15 (18.75)	
Education levels, n (%)			
Graduate school	4 (10.00)	5 (6.25)	
College (or junior college)	19 (47.50)	24 (30.00)	
Senior (Vocational) high school	10 (25.00)	25 (31.25)	0.330 [b]
Junior high school	2 (5.00)	10 (12.50)	
Elementary school	5 (12.50)	15 (18.75)	
No education	0 (0.00)	1 (1.25)	
Annual household income, n (%)			
Above 2 millions	0 (0.00)	1 (1.25)	
1.5~2 millions	0 (0.00)	1 (1.25)	
1.0~1.5 millions	1 (2.50)	1 (1.25)	0.186 [c]
0.5~1.0 million	7 (17.50)	4 (5.00)	
Less than 0.5 million	32 (80.00)	73 (91.25)	
Medical history, n (%)			
Yes	29 (72.50)	50 (62.50)	0.276 [b]
No	11 (27.50)	30 (37.50)	

[a] Statistical results were determined by using Mann Whitney test

[b] Statistical results were determined by using Chi square (X^2) test

[c] Statistical results were determined by using Fisher exact test

The medical history were listed in the appendix II

Table 5. Descriptive statistics of lifestyle on demographic characteristics of subjects

Variable	NS (n=40) [a]	SC (n=80) [a]	*p*-value
Daily personal habits			
Drinking coffee, n (%)			
Yes	23 (57.50)	34 (42.50)	0.121 [b]
No	17 (42.50)	46 (57.50)	
Drinking tea, n (%)			
Yes	16 (40.00)	21 (26.25)	0.124 [b]
No	24 (60.00)	59 (73.75)	
Drinking alcohol, n (%)			
Yes	5 (12.50)	1(1.25)	0.015 [*c]
No	35 (87.50)	79 (98.75)	
Vitamin supplement, n (%)			
Yes	32 (80.00)	57 (71.25)	0.302 [b]
No	8 (20.00)	23 (28.75)	
Oil-smoke exposure assessment			
Oil fume exposure every day, n (%)			
Yes	27 (67.50)	49 (61.25)	0.503 [b]
No	13 (32.50)	31 (38.75)	
Utilizing a range hood at home, n (%)			
Yes			
a. Always utilized (≧3 times/week)	33 (82.50)	59 (73.75)	0.106 [b]
b. Rarely utilized (≦3 times/week)	3 (7.50)	2 (2.50)	
No	4 (10.00)	19 (23.75)	
Utilizing incense at home, n (%)			
Yes			
a. ≦ 1 time/month	0 (0.00)	3 (3.75)	0.576 [b]
b. ≧ 1 time/month -≦1 time/week	1 (2.50)	2 (2.50)	
c. ≧ 1 time/week-≦1 time/day	0 (0.00)	2 (2.50)	
d. ≧ 1 time/day	5 (12.50)	7 (8.75)	
No	34 (85.00)	66 (82.50)	
Utilizing mosquito coil at home, n (%)			
Yes			0.028 [* b]
a. ≦ 1 time/month	2 (5.00)	0 (0.00)	

b. ≧ 1 time/month -≦1 time/week	0 (0.00)	1 (1.25)	
c. ≧ 1 time/week-≦1 time/day	1 (2.50)	0 (0.00)	
d. ≧ 1 time/day	2 (5.00)	0 (0.00)	
No	35 (87.50)	79 (98.75)	
Cigarette smoking habit, n (%)			
Yes	1 (2.50)	2 (2.50)	1.000[c]
No	39 (97.50)	78 (97.50)	
Secondhand smoke exposure, n (%)			
Yes	0 (0.00)	8 (10.00)	0.051[c]
No	40 (100.00)	72 (90.00)	

[a] Data are presented as N (%)

[b] Statistical results were determined by using Pearson chi square (X^2) test

[c] Statistical results were determined by using Fisher exact test

*p<0.05

Table 6. Comparison of body composition between the NS and SC groups

Parameter	NS (n=40) [a]	SC (n=80) [a]	*p*-value [b]
Body composition			
Calf circumference (cm)	35.25 (3.00)	31.55 (2.43)	0.000***
Body height (cm)	156 (8.8)	154 (9.0)	0.064#
Body weight (kg)	58.65 (13.58)	49.00 (8.00)	0.000***
BMI (kg/m^2)	24.23 (2.93)	20.80 (2.96)	0.000***
SMI (kg/m^2)	6.35 (0.90)	5.4 (0.58)	0.000***
Skeletal muscle mass (kg)	20.95 (4.28)	17.60 (2.62)	0.000***
Soft lean mass (kg)	36.2 (6.75)	31.4 (3.88)	0.000***
Fat free mass (kg)	38.55 (7.02)	33.35 (4.50)	0.000***
Protein (kg)	7.55 (1.40)	6.50 (0.98)	0.000***
Fat (kg)	18.80 (5.93)	15.30 (6.10)	0.000***
Intracellular water (kg)	17.60 (3.27)	15.00 (2.02)	0.000***
Extracellular water (kg)	11.10 (1.85)	9.50 (1.18)	0.000***
Total body water (kg)	28.35 (5.17)	24.50 (2.97)	0.000***
Whole body phase angle (°)	5.20 (1.18)	4.60 (0.58)	0.000***

[a] Data were presented as median (IQR)

[b] Statistical results were determined by using Mann Whitney test; # $p<0.1$, *$p<0.05$, **$p<0.01$, ***$p<0.001$

Table 7. Comparison of physical performance between the NS and SC groups

Parameter	NS (n=40) [a]	SC (n=80) [a]	*p*-value [b]
Physical performance			
Handgrip (kg)	22.17 (9.31)	17.84 (7.02)	0.000***
Chair stand test (sec)	12.91 (5.51)	14.02 (5.37)	0.149
Walk speed (m/s)	0.893 (0.307)	0.843 (0.324)	0.412
TUG test (sec)	10.12 (3.90)	11.38 (4.09)	0.152
SPPB score (pt)	11 (3.75)	10 (3.00)	0.005**

[a] Data were presented as median (IQR)

[b] Statistical results were determined by using Mann Whitney test; # $p<0.1$, *$p<0.05$, **$p<0.01$, ***$p<0.001$

Table 8. Comparison of physical activity by IPAQ between NS and SC groups

Physical activity	NS group (n=40) [a]	SC group (n=80) [a]	Chi-square (χ^2)	*p*-value [b]
High	14 (35.00)	24 (30.00)		
Medium	17 (42.50)	44 (55.00)	1.887	0.389
Low	9 (22.50)	12 (15.00)		

[a] Data are presented as N (%)

[b] *p*-value was determined by using Pearson chi square (X^2) test

Table 9. Comparison of nutritional assessment by MNA score between NS and SC groups

MNA score	NS group (n=40) [a]	SC group (n=80) [a]	Chi-square (χ^2)	*p*-value [b]
Well-nourished	40 (100.00)	66 (82.50)		
At risk of malnourished	0 (0.00)	14 (17.50)	7.925	0.002**
Malnourished	0 (0.00)	0 (0.00)		

[a] Data are presented as N (%)

[b] *p*-value was determined by using Fisher's exact test (two-tailed)

*p<0.05, **p<0.01.

Table 10. Comparison of nutritional assessment by 24 hour-recall methods between NS and SC groups

24 hour-recall methods	NS group (n=40) [a]	SC group (n=80) [a]	*p*-value [b]
Total calories (kcal)	1260 (399)	1251 (449)	0.717
Carbohydrate (%)	50.76 (14.80)	47.63 (14.10)	0.758
Protein (%)	18.59 (5.26)	17.03 (6.10)	0.202
Fat (%)	31.96 (13.37)	33.97 (12.19)	0.496

[a] Data are presented as median (IQR)

[b] Statistical results were determined by using Mann Whitney test; *p<0.05, **p<0.01, ***p<0.001

Table 11. Descriptive statistics of Food frequency ingestion on demographic characteristics of subjects

Variable	NS (n=40) [a]	SC (n=80) [a]	*p*-value [b]
Food frequency ingestion			
Fish ingestion			
The frequency of fish is ingested (times/month)	8.00 (14.00)	6.00 (12.38)	0.270
The frequency with which deep fish is ingested (times/month)	4.00 (6.88)	4.00(8.00)	0.652
The frequency at which other seafood is ingested (times/month)	5.50 (12.26)	3.00 (6.00)	0.019*
Meat ingestion			
The frequency with which meat is ingested (times/month)	28.50 (31.75)	23.50 (24.13)	0.151
Animal source of viscous of blood ingestion			
The frequency with which animal source of viscera or blood is ingested (times/month)	0.00 (1.00)	0.00 (0.00)	0.016*
Meat processing product			
The frequency with which meat processing products are ingested (times/month)	2.00 (4.38)	1.00 (4.75)	0.032*
Soybean ingestion			
The frequency with which soybean products are ingested (times/month)	9.25 (12.75)	9.50 (15.50)	0.825

[a] Data are presented as median (IQR)

[b] Statistical results were determined by using Mann Whitney test

*$p<0.05$

Table 12. Comparison severity of constipation symptoms by PAC-SYM between the NS and SC groups

PAC-SYM	NS group (n=40) [a]	SC group (n=80) [a]	*p*-value [b]
Abdominal	0 (1)	0 (1)	0.290
Rectal	0 (1.75)	0 (1)	0.920
Stools	0 (1)	0 (0)	0.155
Total	1 (4.75)	1 (3)	0.454

[a] Data are presented as median (IQR)

[b] Statistical results were determined by using Mann Whitney test; *$p<0.05$, **$p<0.01$, ***$p<0.001$

Table 13. Comparison of biochemical parameters between NS and SC groups

Biochemical parameter	NS group (n=40) [a]		SC group (n=80) [a]		Chi-square (χ^2)	*p*-Value
	Normal	Abnormal	Normal	Abnormal		
TSH	39 (97.50)	1 (2.50)	77 (96.25)	3 (3.75)	0.129	1.000 [c]
Free T4	38 (95.00)	2 (5.00)	72 (90.00)	8 (10.00)	0.873	0.493 [c]
hsCRP (mg/dL)	40 (100.00)	0 (0.00)	79 (98.75)	1 (1.25)	0.504	1.000 [c]
Albumin (g/dL)	40 (100.00)	0 (0.00)	78 (97.50)	2 (2.50)	1.017	0.552 [c]
Pre-albumin (mg/dL)	38 (95.00)	2 (5.00)	78 (97.50)	2 (2.50)	0.104	1.000 [c]
CHOL (mg/dL)	32 (80.00)	8 (20.00)	47 (58.75)	33 (41.25)	1.440	0.230 [b]
HDL-C (mg/dL)	30 (75.00)	10 (25.00)	69 (86.25)	11 (13.75)	2.338	0.126 [b]
LDL-C (mg/dL)	22 (55.00)	18 (45.00)	35 (43.75)	45 (56.25)	1.353	0.245 [b]
TG (mg/dL)	33 (82.50)	7 (17.50)	70 (87.50)	10 (12.50)	0.548	0.459 [b]
HbA1c (%)	14 (35.00)	26 (65.00)	34 (42.50)	46 (57.50)	0.625	0.429 [b]
GLU(AC) (mg/dL)	23 (57.50)	17 (42.50)	47 (58.75)	33 (41.25)	0.017	0.896 [b]
Insulin (uIU/mL)	38 (95.00)	2 (5.00)	79 (98.75)	1 (1.25)	1.538	0.257 [c]
HOMA-IR	16 (40.00)	24 (60.00)	37 (46.25)	43 (53.75)	0.422	0.516 [b]
AST (U/L)	40 (100.00)	0 (0.00)	77 (96.25)	3 (3.75)	1.538	0.550 [c]
ALT (U/L)	40 (100.00)	0 (0.00)	77 (96.25)	3 (3.75)	1.538	0.550 [c]
BUN (mg/dL)	34 (85.00)	6 (15.00)	65 (81.25)	15 (18.75)	0.260	0.610 [b]
Creatinine (mg/dL)	40 (100.00)	0 (0.00)	80 (100.00)	0 (0.00)	-	-
eGFR (mL/min/1.73 m^2)	37 (92.50)	3 (7.50)	74 (92.50)	6 (7.50)	0.000	1.000 [c]
WBC (1000/μl)	40 (100.00)	0 (0.00)	80 (100.00)	0 (0.00)	-	-
RBC (million/μl)	40 (100.00)	0 (0.00)	80 (100.00)	0 (0.00)	-	-
HGB (g/dL)	40 (100.00)	0 (0.00)	80 (100.00)	0 (0.00)	-	-
HCT (%)	40 (100.00)	0 (0.00)	80 (100.00)	0 (0.00)	-	-
MCHC (g/dL)	40 (100.00)	0 (0.00)	79 (98.75)	1 (1.25)	0.504	1.000 [c]
Platelet (1000/uL)	35 (87.50)	5 (12.50)	74 (92.50)	6 (7.50)	0.801	0.503 [c]
RDW-CV (%)	33 (82.50)	7 (17.50)	73 (91.25)	7 (8.75)	1.981	0.226 [c]

[a] Data are presented as N (%)

[b] *p*-value was determined by using Chi-square ($\chi 2$) test (two-tailed)

[c] *p*-value was determined by using Fisher's exact test (two-tailed)

Table 14. Comparison of different T lymphocyte types between the NS and SC groups.

Parameter	NS (n=40) [a]	SC (n=80) [a]	*p*-value [b]
$CD3^+$ T cell (%)	39.39 (17.38)	43.09 (20.01)	0.401
$CD4^+$ T cell (%)	42.37 (16.52)	51.06 (27.31)	0.044*
$CD8^+$ T cell (%)	28.51 (17.31)	32.47 (17.62)	0.183
Regulatory T lymphocyte (%)	0.081 (0.264)	0.125 (0.136)	0.298

[a] Data were presented as median (IQR)

[b] Statistical results were determined by using Mann Whitney test; *$p<0.05$

Table 15. Comparison of Pro-inflammatory and anti-inflammatory cytokines between NS and SC groups

Parameter	NS (n=40) [a]	SC (n=80) [a]	*p*-value [b]
IL-6 (pg/mL)	0.47 (1.04)	7.64 (20.48)	0.000***
IL-10 (pg/mL)	3.45 (3.73)	0.50 (3.94)	0.001**
IL-17 (pg/mL)	32.25 (106.22)	9.38 (51.58)	0.014*
TNF-α (pg/mL)	5.97 (9.81)	12.63 (56.05)	0.014*
TGF-β (ng/mL)	22.52 (9.10)	6.36 (15.52)	0.000***

[a] Data are presented as median (IQR)

[b] Statistical results were determined by using Mann Whitney test ; $^{\#}p<0.1$, *p<0.05, **p<0.01, ***p<0.001

Table 16. Comparison of antioxidant activity between NS and SC groups

Parameter	NS (n=40) [a]	SC (n=80) [a]	*p*-value [b]
CAT (U/mL)	38.84 (18.97)	29.82 (30.55)	0.028*
GPx (U)	111.30 (17.70)	98.87 (30.85)	0.004**
SOD (U/mL)	191.3 (12.0)	188.6 (115.1)	0.514

[a] Data are presented as median (IQR)

[b] Statistical results were determined by using Mann Whitney test ; # *p<0.1*, *$p<0.05$, **$p<0.01$, ***$p<0.001$

Table 17. Comparison of anabolic hormone between NS and SC groups

Parameter	NS (n=40) [a]	SC (n=80) [a]	*p*-value [b]
DHEA-S (μg/mL)	107.80 (82.19)	90.57 (62.45)	0.082 [#]

[a] Data are presented as median (IQR)

[b] Statistical results were determined by using Mann Whitney test ; [#] *p<0.1*, *$p<0.05$, **$p<0.01$, ***$p<0.001$

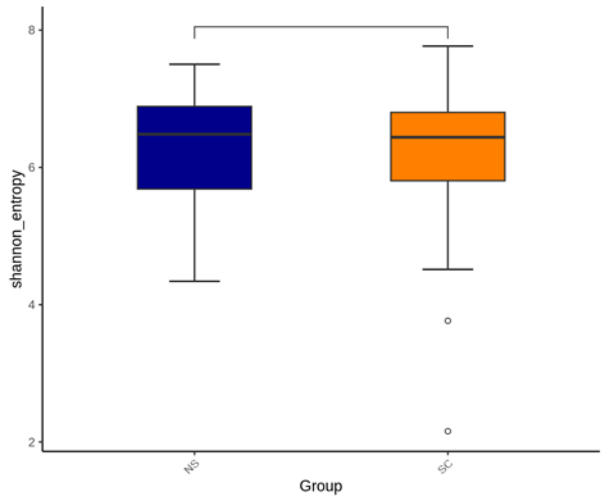
Table 18. Comparison of variety metals level in the serum between NS and SC groups

Metal (μg/L)	NS (n=36) [a]	SC (n=78) [a]	*p*-value [b]
Na	2840438 (496896)	2730763 (498577)	0.118
Mg	43673(7005)	43277 (8358)	0.897
K	2724861 (514371)	2813224(613906)	0.223
Mn	11.45 (4.28)	11.35 (5.18)	0.946
Fe	406895 (95802)	397900 (70831)	0.714
Ni	1.42 (0.67)	1.80 (1.02)	0.006**
Cu	752.0 (119.7)	761.0 (136.0)	0.737
Zn	6298 (1961)	6103 (1394)	0.209
Sr	27.80 (9.18)	21.42 (10.55)	0.548
As	6.35 (8.59)	5.91 (5.69)	0.670
Se	403.0 (90.7)	365.4 (96.9)	0.007**
Mo	1.15 (0.84)	0.97 (0.67)	0.699
Cd	0.56 (0.31)	0.59 (0.33)	0.562
Sb	4.38 (1.05)	5.19 (2.63)	0.019*
Ba	0.93 (0.67)	0.89 (0.53)	0.644
Pb	17.03 (9.93)	14.14 (9.88)	0.045*

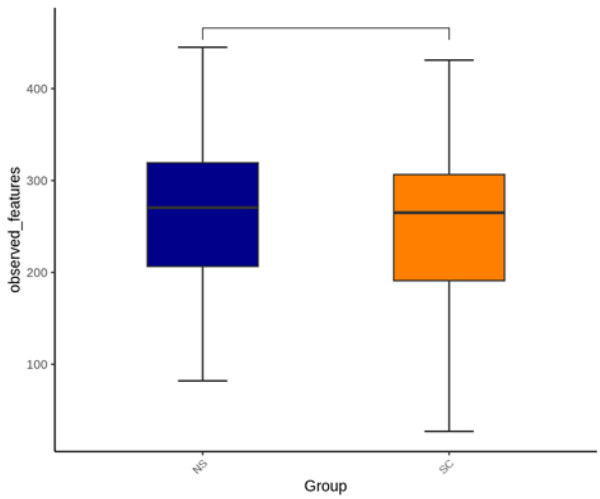
[a] Data were presented as median (IQR)

[b] Statistical results were determined by using Mann Whitney test; *p<0.05, **p<0.01

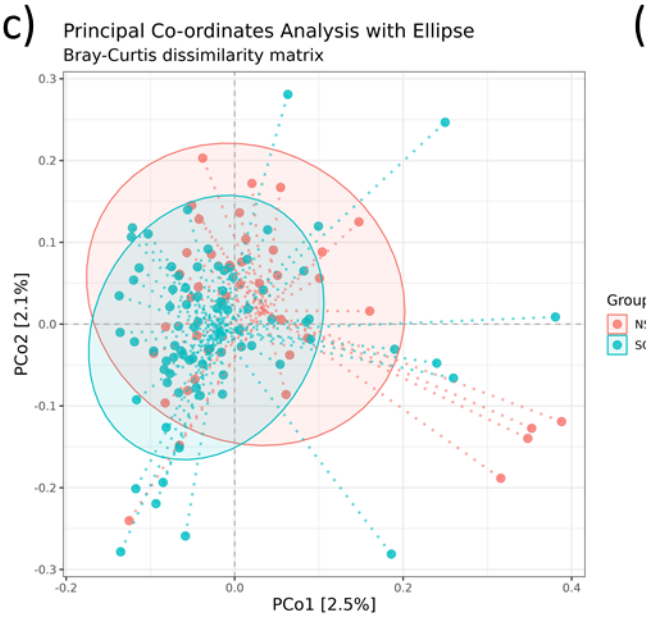
(a)


shannon_entropy
8
6
4
2
NS
SC
Group

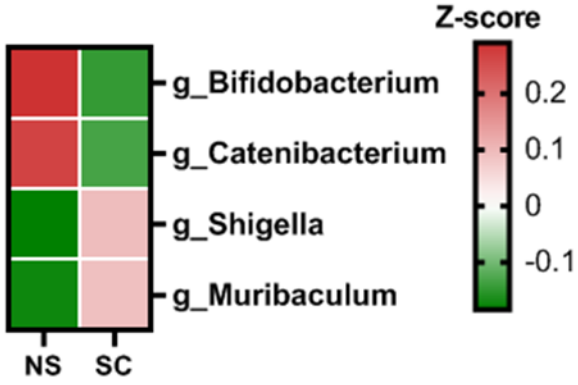
(b)


observed_features
400
300
200
100
NS
SC
Group

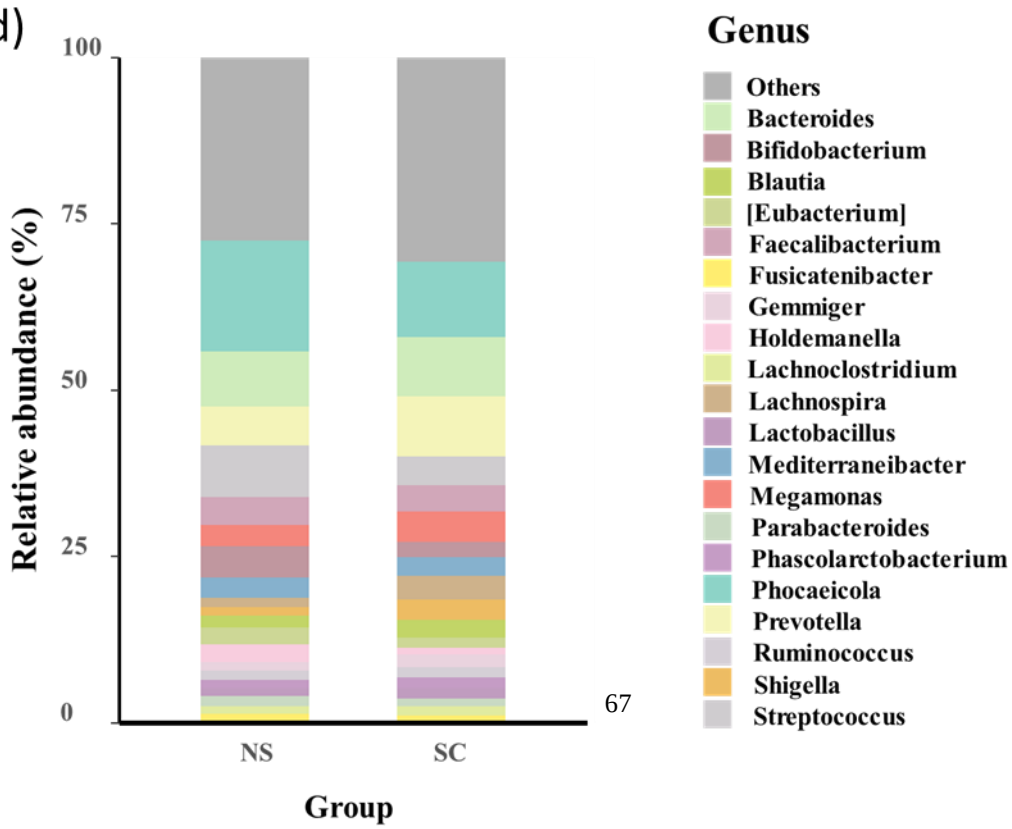
(c)


Principal Co-ordinates Analysis with Ellipse
Bray-Curtis dissimilarity matrix
PCo2 [2.1%]
PCo1 [2.5%]
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.2
0.0
0.2
0.4
Group

(e)


Z-score
0.2
0.1
0
-0.1
g_Bifidobacterium
g_Catenibacterium
g_Shigella
g_Muribaculum
NS
SC

(d)


Relative abundance (%)
100
75
50
25
0
NS
SC
Group

Genus
Others
Bacteroides
Bifidobacterium
Blautia
[Eubacterium]
Faecalibacterium
Fusicatenibacter
Gemmiger
Holdemanella
Lachnoclostridium
Lachnospira
Lactobacillus
Mediterraneibacter
Megamonas
Parabacteroides
Phascolarctobacterium
Phocaeicola
Prevotella
Ruminococcus
Shigella
Streptococcus

(f)

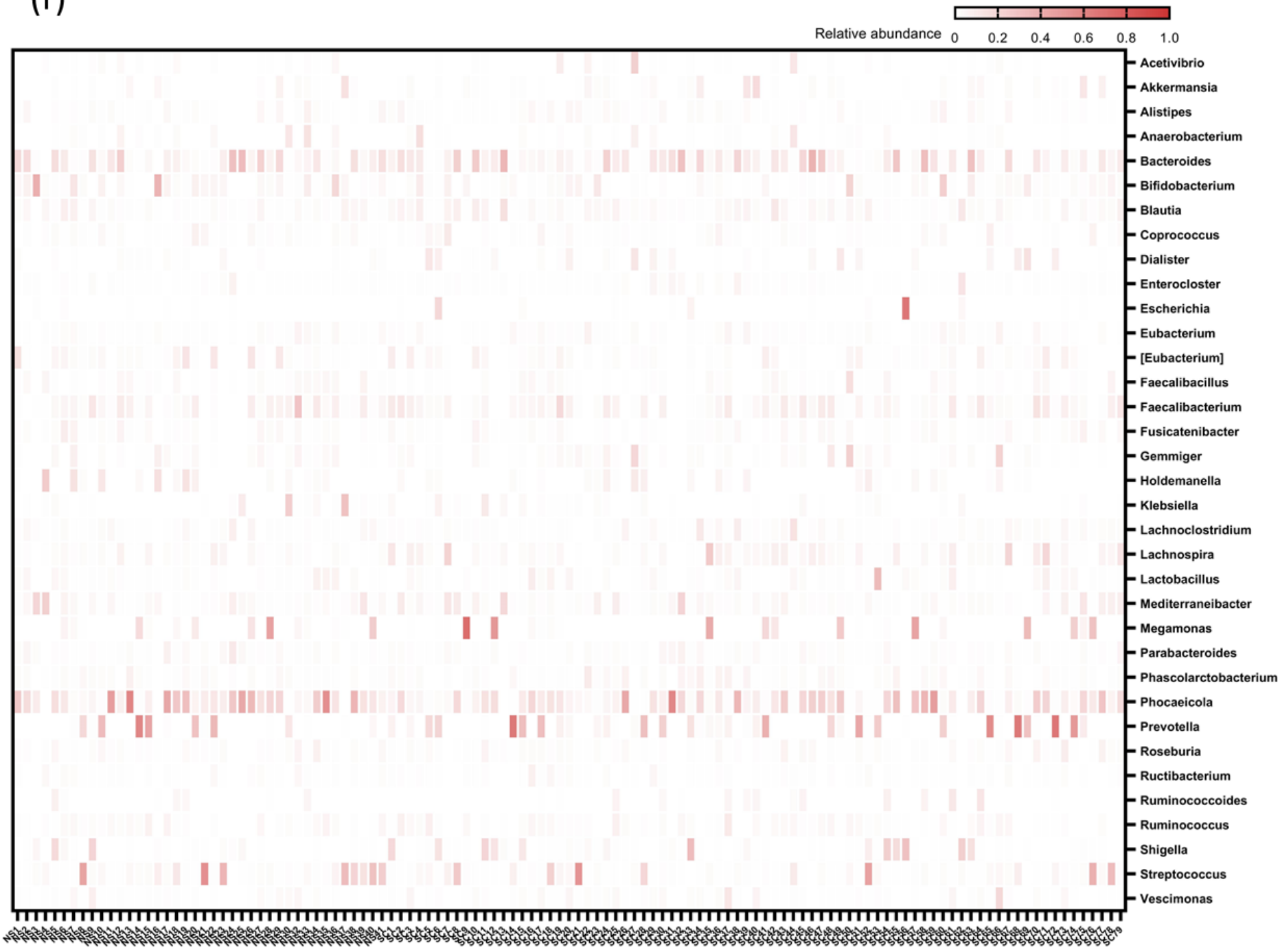

Relative abundance
0
0.2
0.4
0.6
0.8
1.0
Acetivibrio
Akkermansia
Alistipes
Anaerobacterium
Bacteroides
Bifidobacterium
Blautia
Coprococcus
Dialister
Enterocloster
Escherichia
Eubacterium
[Eubacterium]
Faecalibacillus
Faecalibacterium
Fusicatenibacter
Gemmiger
Holdemanella
Klebsiella
Lachnoclostridium
Lachnospira
Lactobacillus
Mediterraneibacter
Megamonas
Parabacteroides
Phascolarctobacterium
Phocaeicola
Prevotella
Roseburia
Ructibacterium
Ruminococcoides
Ruminococcus
Shigella
Streptococcus
Vescimonas

Figure 3. Alterations of gut microbiota composition between control group (NS) and Sarcopenia Control (SC).

(A & B) Box-and-whisker plots show α-diversity indices (Shannon index and Observed features index) comparing NS and SC groups. (C) β-diversity assessed using Bray-Curtis distance and visualized by PCoA. (D) Bar plot showing the relative abundance of the top 20 bacterial genera. (E) Heatmap showing taxa with significant linear discriminant analysis (LDA) scores (>3.0), indicating genera that are differentially abundant between SC and LcS, as identified by LEfSe. The heatmap displays Z-score–normalized relative abundance values. For each genus, the relative abundance was averaged within each group and then standardized using Z-score transformation across groups. Deep red indicates higher relative abundance; deep green indicates lower abundance. Only genus-level taxa are shown, and they are presented in alphabetical order for clarity and consistency with the main text. (F) Heatmap showing the relative abundance of the top 35 gut microbiota genera across individual subjects. Each vertical column represents one subject, and the color intensity indicates the abundance level. Red denotes higher relative abundance.

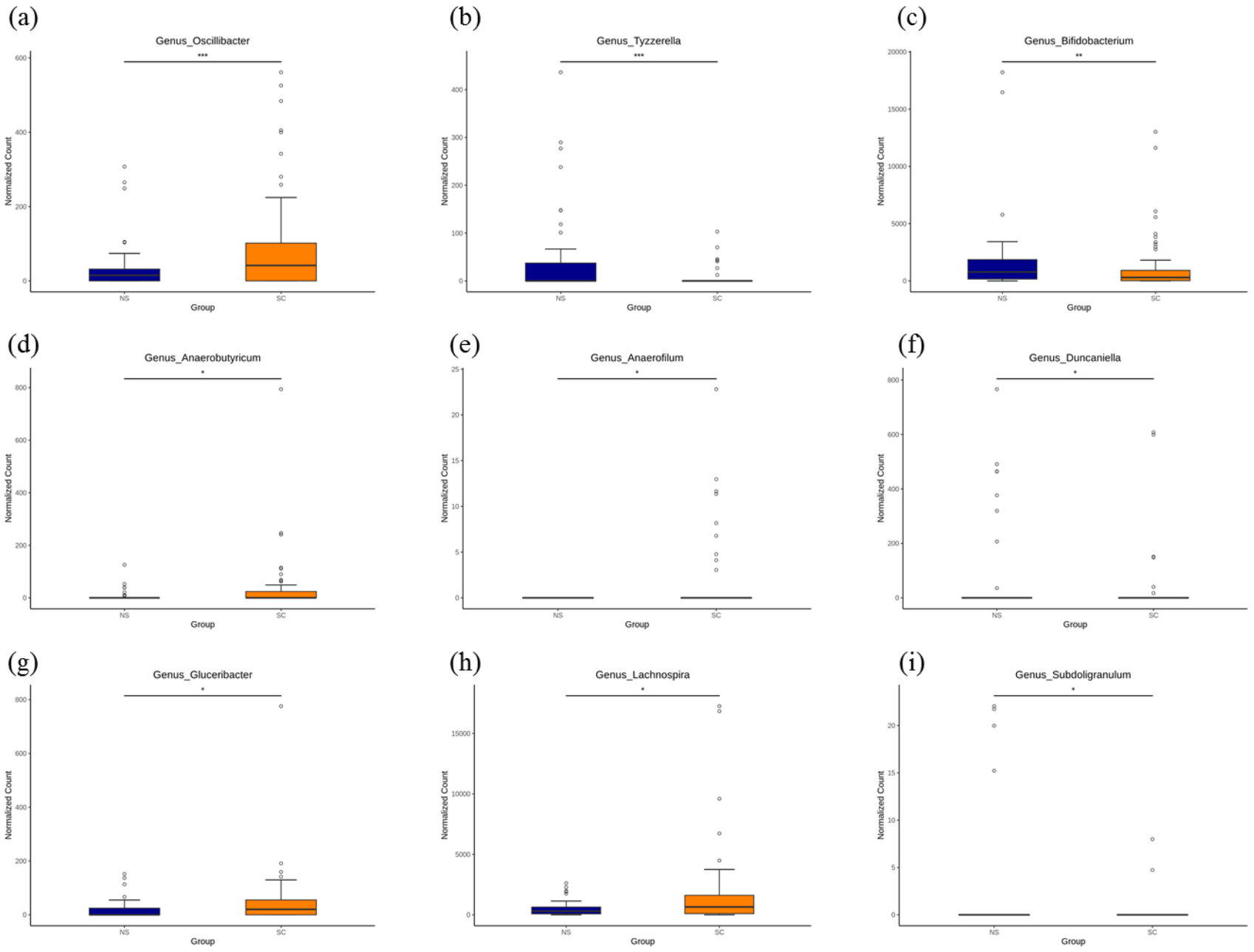

(a)
Genus_Oscillibacter
***
Normalized Count
Group
NS
SC
(b)
Genus_Tyzzerella
***
Normalized Count
Group
NS
SC
(c)
Genus_Bifidobacterium
**
Normalized Count
Group
NS
SC
(d)
Genus_Anaerobutyricum
*
Normalized Count
Group
NS
SC
(e)
Genus_Anaerofilum
*
Normalized Count
Group
NS
SC
(f)
Genus_Duncaniella
*
Normalized Count
Group
NS
SC
(g)
Genus_Gluceribacter
*
Normalized Count
Group
NS
SC
(h)
Genus_Lachnospira
*
Normalized Count
Group
NS
SC
(i)
Genus_Subdoligranulum
*
Normalized Count
Group
NS
SC

Figure 4. MetagenomeSeq analysis of gut microbiota compared NS and SC group.

Bacterial genus that were significantly more abundant between NS and SC group with high adherence (FDR < 0.05) by metagenomeSeq test, including (a)Oscillibacter, (b)Tyzzerella, (c)Bifidobacterium, (d)Anaerobutyricum, (e)Anaerofilum, (f)Duncaniella, (g)Gluceribacter, (h)Lachnospira, and (i)Subdoligranulum.

Table 19. Differentially Abundant Genera Between NS and SC Identified by LEfSe and MetagenomeSeq

Category	Increased	Decreased
(1) Both LEfSe and MetagenomeSeq		*Bifidobacterium*
(2) LEfSe only	*Shigella, Muribaculum*	*Catenibacterium*
(3) MetagenomeSeq only	*Anaerobutyricum, Anaerofilum, Gluceribacter, Lachnospira, Oscillibacter*	*Duncaniella, Subdoligranulum, Tyzzerella*

The direction of change is indicated relative to the NS group, with genera classified as significantly increased or decreased in the SC group. Results are categorized based on detection by (1) both LEfSe and MetagenomeSeq, (2) LEfSe only, or (3) MetagenomeSeq only.

Influence of Fermented Milk Containing *Lactobacillus casei* Strain Shirota intervention:

Table 20 Comparison of the results of anthropometric measurements between pre- and post-intervention in the SC and LcS groups using bioelectrical impedance analysis (BIA).

	SC (n=40) [a]			LcS (n=40) [a]			SC v.s. LcS (Post-test)
Parameter	Pre-test	Post-test	*p*-value [b]	Pre-test	Post-test	*p*-value [b]	*p*-value [c]
Calf circumference (cm)	32.00 (3.15)	32.00 (2.65)	0.457	31.50 (1.90)	31.50 (2.00)	0.718	0.289
Body height (cm)	154.3 (16.3)	154.3 (16.3)	0.250	153.0 (9.0)	153.5 (8.8)	0.987	0.473
Body weight (kg)	49.00 (8.00)	49.50 (9.63)	0.935	49.00 (8.35)	49.00 (6.83)	0.876	0.360
BMI (kg/m^2)	20.84 (3.95)	20.85 (3.29)	0.836	20.69 (2.67)	20.89 (2.72)	0.798	0.567
SMI (kg/m^2)	5.40 (0.55)	5.55 (0.75)	0.027*	5.30 (0.50)	5.40 (0.70)	0.169	0.078
Skeletal muscle mass (kg)	17.55 (2.80)	17.80 (3.38)	0.275	17.60 (2.45)	17.75 (2.57)	0.362	0.308
Soft lean mass (kg)	31.30 (4.67)	31.70 (4.45)	0.997	31.4 (3.67)	31.85 (3.83)	0.113	0.473
Fat free mass (kg)	33.45 (4.85)	33.75 (5.75)	0.593	33.25 (3.98)	33.90 (4.03)	0.134	0.256
Protein (kg)	6.45 (0.98)	6.50 (1.15)	0.285	6.50 (0.88)	6.60 (0.95)	0.285	0.429
Fat (kg)	15.15 (6.97)	15.10 (6.95)	0.480	15.45 (4.45)	15.05 (5.42)	0.167	0.851
Intracellular water (kg)	14.95 (2.12)	15.15 (2.18)	0.157	15.00 (1.95)	15.15 (2.15)	0.235	0.301
Extracellular water (kg)	9.55 (1.50)	9.65 (1.68)	0.052	9.45 (1.18)	9.65 (1.43)	0.182	0.389
Total body water (kg)	24.45 (3.77)	24.75 (3.97)	0.004**	24.5(2.92)	24.90 (2.92)	0.087	0.248
Whole body phase angle (°)	4.50 (0.48)	4.70 (0.58)	0.047*	4.50 (0.68)	4.45 (0.68)	0.763	0.060

[a] Data were presented as median (IQR)

[b] Statistical results were determined by using Wilcoxon matched-pairs signed rank test; *$p<0.05$, **$p<0.01$

[c] Statistical results were determined by using Mann Whitney test

Table 21. Comparison of physical performance between pre- and post-intervention in the SC and LcS groups.

	SC (n=40) [a]			LcS (n=40) [a]			SC v.s. LcS (Post-test)
Parameter	Pre-test	Post-test	*p*-value [b]	Pre-test	Post-test	*p*-value [b]	*p*-value [c]
Handgrip (kg)	17.77 (7.31)	18.95 (6.10)	0.169	17.84 (5.80)	19.07 (6.64)	0.004**	0.989
Chair stand test (sec)	14.39 (5.31)	14.95 (6.69)	0.319	13.90 (5.31)	12.31 (6.39)	0.027*	0.003**
Walk speed (m/s)	0.830 (0.336)	0.825 (0.245)	0.191	0.860(0.296)	1.077(0.408)	0.038*	0.009**
TUG test (sec)	12.14 (4.63)	12.05 (5.01)	0.727	11.96 (3.99)	9.06 (4.96)	0.001**	0.170
SPPB score (pt)	10 (6.00)	9 (6.00)	0.310	10 (3.00)	11 (3.00)	0.006**	0.015*

[a] Data were presented as median (IQR)

[b] Statistical results were determined by using Wilcoxon matched-pairs signed rank test; *p<0.05, **p<0.01

[c] Statistical results were determined by using Mann Whitney test; *p<0.05, **p<0.01

Table 22. Comparison of pre- and post-intervention physical activity using IPAQ between SC and LcS groups

Physical activity	SC group (n=40) [a]			LcS group (n=40) [a]			Post-test (SC v.s. LcS)	
	Pre-test (n=40)	Post-test (n=40)	*p*-value [b]	Pre-test (n=40)	Post-test (n=40)	*p*-value [b]	Chi-square (χ^2)	*p*-value [c]
High	10 (25)	9 (22.5)		14 (35)	13 (32.5)			
Medium	23 (57.5)	25 (62.5)	0.392	21 (52.5)	20 (50)	0.557	1.360	0.507
Low	7 (17.5)	6 (15)		5 (12.5)	7 (17.5)			

[a] Data are presented as N (%)

[b] *p*-value was determined by using Mcnemar's test

[c] *p*-value was determined by using Pearson chi square (X^2) test

Table 23. Comparison of physical activity pre- and post-intervention using MNA between SC and LcS groups.

MNA score	SC group (n=40) [a]		*p*-value [b]	LcS group (n=40) [a]		*p*-value [b]	Post-test (SC v.s. LcS)	
	Pre-test (n=40)	Post-test (n=40)		Pre-test (n=40)	Post-test (n=40)		Chi-square (χ^2)	*p*-value [c]
Well-nourished	34 (85.00)	37 (92.50)		32 (80.00)	38 (95.00)			
At risk of malnourished	6 (15.00)	3 (7.50)	0.375	8 (20.00)	2 (5.00)	0.031*	0.213	1.000
Malnourished	0 (0.00)	0 (0.00)		0 (0.00)	0 (0.00)			

[a] Data are presented as N (%)

[b] *p*-value was determined by using Mcnemar's test; **p*<0.05

[c] *p*-value was determined by using Fisher's exact test (two-tailed)

Table 24. Comparison of nutritional assessment using 24-hour recall methods pre- and post-intervention, and between SC and LcS groups.

24 hour-recall methods	SC group (n=40) [a]		*p*-value [c]	LcS group (n=40) [ab]		*p*-value [c]	Post-test (SC v.s. LcS)
	Pre-test	Post-test		Pre-test	Post-test		*p*-value [d]
Total calories (kcal)	1296 (460)	1400 (476)	0.281	1228 (427)	1257 (395)	0.248	0.273
Carbohydrate (%)	168.6 (76.5)	179.3 (75.5)	0.261	139.5 (61.9)	160.5 (45.2)	0.056	0.373
Protein (%)	53.35 (21.92)	55.50 (21.50)	0.981	58.15 (31.68)	57.53 (27.27)	0.065	1.000
Fat (%)	48.7 (26.28)	51.25 (36.37)	0.381	47 (22.03)	51.75 (24.73)	0.381	0.387

[a] Data are presented as median (IQR)

[b] Data include the nutrient content from Yakult 300 LIGHT

[c] Statistical results were determined by using Wilcoxon matched-pairs signed rank test

[d] Statistical results were determined by using Mann Whitney test

Table 25. Comparison of constipation symptom severity using PAC-SYM for pre- and post-intervention, and between SC and LcS groups.

PAC-SYM	SC group (n=40) [a]		*p*-value [b]	LcS group (n=40) [a]		*p*-value [b]	Post-test (SC v.s. LcS)
	Pre-test	Post-test		Pre-test	Post-test		*p*-value [c]
Abdominal	0 (1)	0 (1)	0.842	0 (1)	0 (0)	0.049*	0.024*
Rectal	0 (1)	0 (2)	0.704	0 (1)	0 (1)	0.379	0.484
Stools	0 (0)	0 (0)	0.844	0 (0.75)	0 (0)	0.220	0.883
Total	1 (3.75)	1 (3.75)	0.709	1 (3.00)	0 (1.00)	0.035*	0.192

[a] Data are presented as median (IQR)

[b] Statistical results were determined by using Wilcoxon matched-pairs signed rank test; *$p<0.05$

[c] Statistical results were determined by using Mann Whitney test; *$p<0.05$

Table 26. Comparison of biochemical parameters Pre and Post intervention in the SC group.

Biochemical parameter	SC group (n=40) [a]				*p*-Value [b]
	Pretest (n=40)		Posttest (n=40)		
	Normal	Abnormal	Normal	Abnormal	
TSH	38 (95)	2 (5)	39 (97.5)	1 (2.5)	1
Free T4	32 (80)	8 (20)	31 (77.5)	9 (22.5)	1
hsCRP (mg/dL)	39 (97.5)	1 (2.5)	39 (97.5)	1 (2.5)	1
Albumin (g/dL)	40 (100)	0 (0)	40 (100)	0 (0)	-
Pre-albumin (mg/dL)	40 (100)	0 (0)	39 (97.5)	1 (2.5)	-
CHOL (mg/dL)	25 (62.5)	15 (37.5)	27 (67.5)	13 (32.5)	0.5
HDL-C (mg/dL)	34 (85)	6 (15)	33 (82.5)	7 (17.5)	1
LDL-C (mg/dL)	17 (42.5)	23 (57.5)	21 (52.5)	19 (47.5)	0.289
TG (mg/dL)	37 (92.5)	3 (7.5)	38 (95)	2 (5)	1
HbA1c (%)	18 (45)	22 (55)	18 (45)	22 (55)	1
GLU(AC) (mg/dL)	20 (50)	20 (50)	21 (52.5)	19 (47.5)	1
Insulin (uIU/mL)	40 (100)	0 (0)	39 (97.5)	1 (2.5)	-
HOMA-IR	27 (67.5)	13 (32.5)	30 (75)	10 (25)	0.375
AST (U/L)	39 (97.5)	1 (2.5)	40 (100)	0 (0)	-
ALT (U/L)	39 (97.5)	1 (2.5)	40 (100)	0 (0)	-
BUN (mg/dL)	31 (77.5)	9 (22.5)	32 (80)	8 (20)	1
Creatinine (mg/dL)	38 (95)	2 (5)	38 (95)	2 (5)	1
eGFR (mL/min/1.73 m^2)	37 (92.5)	3 (7.5)	37 (92.5)	3 (7.5)	1
WBC (1000/μl)	37 (92.5)	3 (7.5)	37 (92.5)	3 (7.5)	1
RBC (million/μl)	26 (65)	14 (35)	26 (65)	14 (35)	1
HGB (g/dL)	36 (90)	4 (10)	37 (92.5)	3 (7.5)	1
HCT (%)	29 (72.5)	11 (27.5)	25 (62.5)	15 (37.5)	0.289
MCHC (g/dL)	40 (100)	0 (0)	40 (100)	0 (0)	-
Platelet (1000/uL)	37 (92.5)	3 (7.5)	37 (92.5)	3 (7.5)	1
RDW-CV (%)	38 (95)	2 (5)	39 (97.5)	1 (2.5)	1

[a] Data are presented as N (%)

[b] *p*-value was determined by using McNemar's test

Table 27. Comparison of biochemical parameters Pre and Post intervention in LcS group

Biochemical parameter	LcS group [a]				*p*-Value [b]
	Pretest (n=40)		Posttest (n=40)		
	Normal	Abnormal	Normal	Abnormal	
TSH	39 (97.5)	1 (2.5)	38 (95)	2 (5)	1
Free T4	40 (100)	0 (0)	39 (97.5)	1 (2.5)	-
hsCRP (mg/dL)	40 (100)	0 (0)	39 (97.5)	1 (2.5)	-
Albumin (g/dL)	38 (95)	2 (5)	40 (100)	0 (0)	-
Pre-albumin (mg/dL)	38 (95)	2 (5)	35 (87.5)	5 (12.5)	0.375
CHOL (mg/dL)	22 (55)	18 (45)	19 (47.5)	21 (52.5)	0.549
HDL-C (mg/dL)	36 (90)	4 (10)	36 (90)	4 (10)	1
LDL-C (mg/dL)	18 (45)	22 (55)	12 (30)	28 (70)	0.18
TG (mg/dL)	33 (82.5)	7 (17.5)	33 (82.5)	7 (17.5)	1
HbA1c (%)	16 (40)	24 (60)	15 (37.5)	25 (62.5)	1
GLU(AC) (mg/dL)	27 (67.5)	13 (32.5)	30 (75)	10 (25)	0.453
Insulin (uIU/mL)	39 (97.5)	1 (2.5)	39 (97.5)	1 (2.5)	1
HOMA-IR	30 (75)	10 (25)	33 (82.5)	7 (17.5)	0.508
AST (U/L)	38 (95)	2 (5)	36 (90)	4 (10)	0.5
ALT (U/L)	39 (97.5)	1 (2.5)	38 (95)	2 (5)	1
BUN (mg/dL)	34 (85)	6 (15)	35 (87.5)	5 (12.5)	1
Creatinine (mg/dL)	37 (92.5)	3 (7.5)	37 (92.5)	3 (7.5)	1
eGFR (mL/min/1.73 m^2)	37 (92.5)	3 (7.5)	37 (92.5)	3 (7.5)	1
WBC (1000/μl)	38 (95)	2 (5)	37 (92.5)	3 (7.5)	1
RBC (million/μl)	26 (65)	14 (35)	28 (70)	12 (30)	0.727
HGB (g/dL)	37 (92.5)	3 (7.5)	38 (95)	2 (5)	1
HCT (%)	32 (80)	8 (20)	32 (80)	8 (20)	1
MCHC (g/dL)	39 (97.5)	1 (2.5)	40 (100)	0 (0)	-
Platelet (1000/uL)	37 (92.5)	3 (7.5)	37 (92.5)	3 (7.5)	1
RDW-CV (%)	35 (87.5)	5 (12.5)	36 (90)	4 (10)	1

[a] Data are presented as N (%)

[b] *p*-value was determined by using McNemar's test

Table 28. Comparison of biochemical parameters between post-tests of the SC and LcS groups

Biochemical parameter	Posttest [a]				Chi-square (χ^2)	*p*-Value
	SC group (n=40)		LcS group (n=40)			
	Normal	Abnormal	Normal	Abnormal		
TSH	39 (97.50)	1 (2.50)	38 (95.50)	2 (5.00)	0.346	1.00[c]
Free T4	31 (77.50)	9 (22.50)	39 (97.50)	1 (2.50)	7.314	0.014[*c]
hsCRP (mg/dL)	40 (100)	0 (0)	39 (97.50)	1 (2.50)	1.013	1.00[c]
Albumin (g/dL)	40 (100)	0 (0)	40 (100)	0 (0)	-	-
Pre-albumin (mg/dL)	39 (97.50)	1 (2.50)	35 (87.50)	5 (12.50)	2.883	0.201[c]
CHOL (mg/dL)	27 (67.50)	13 (32.50)	19 (47.50)	21 (52.50)	3.274	0.07[b]
HDL-C (mg/dL)	31 (77.50)	9 (22.50)	36 (90.00)	4 (10.00)	2.296	0.225[c]
LDL-C (mg/dL)	21 (52.50)	19 (47.50)	12 (30.00)	28 (70.00)	4.178	0.041[*b]
TG (mg/dL)	38 (95.00)	2 (5.00)	33 (82.50)	7 (17.50)	3.13	0.154[c]
HbA1c (%)	21 (52.50)	19 (47.50)	19 (47.50)	21 (52.50)	0.464	0.496[b]
GLU(AC) (mg/dL)	21 (52.50)	19 (47.50)	30 (75.00)	10 (25.00)	4.381	0.036[*b]
Insulin (uIU/mL)	39 (97.50)	1 (2.50)	39 (97.50)	1 (2.50)	0.000	1.000[c]
HOMA-IR	21 (52.50)	19 (47.50)	21 (52.50)	19 (47.50)	0.000	1.000[b]
AST (U/L)	40 (100)	0 (0)	36 (90.00)	4 (10.00)	4.211	0.116[c]
ALT (U/L)	40 (100)	0 (0)	38 (95.00)	2 (5.00)	2.051	0.494[c]
BUN (mg/dL)	32 (80.00)	8 (20.00)	35 (87.50)	5 (12.50)	0.827	0.363[b]
Creatinine (mg/dL)	37 (92.50)	3 (7.50)	40 (100)	0 (0)	-	-
eGFR (mL/min/1.73 m^2)	37 (92.50)	3 (7.50)	37 (92.50)	3 (7.50)	0.000	1.000[c]
WBC (1000/μl)	40 (100)	0 (0)	40 (100)	0 (0)	-	-
RBC (million/μl)	40 (100)	0 (0)	40 (100)	0 (0)	-	-
HGB (g/dL)	39 (97.50)	1 (2.50)	40 (100)	0 (0)	-	-
HCT (%)	40 (100)	0 (0)	40 (100)	0 (0)	-	-
MCHC (g/dL)	40 (100)	0 (0)	40 (100)	0 (0)	-	-
Platelet (1000/uL)	37 (92.50)	3 (7.50)	37 (92.50)	3 (7.50)	0.000	1.000[c]
RDW-CV (%)	39 (97.50)	1 (2.50)	36 (90.00)	4 (10.00)	1.92	0.359[c]

[a] Data are presented as N (%)

[b] *p*-value was determined by using Chi-square (χ2)test (two-tailed) ; *p<0.05

[c] *p*-value was determined by using Fisher's exact test (two-tailed); *p<0.05

Table 29. Comparison of T lymphocyte subsets pre- and post-intervention, and between SC and LcS groups using flow cytometry.

	SC (n=40) [a]			LcS (n=40) [a]			SC v.s. LcS (Post-test)
Parameter	Pre-test	Post-test	*p*-value [b]	Pre-test	Post-test	*p*-value [b]	*p*-value [c]
$CD3^+$ T cell (%)	41.50 (20.93)	43.06 (20.79)	0.755	46.52 (20.72)	45.15 (24.83)	0.289	0.148
$CD4^+$ T cell (%)	46.65 (22.12)	45.95 (26.79)	0.715	53.68 (27.27)	42.78 (29.88)	0.050	0.989
$CD8^+$ T cell (%)	31.83 (20.94)	32.20 (20.42)	0.715	32.47 (14.80)	32.97 (18.30)	0.283	0.235
Regulatory T cell (%)	0.124 (0.121)	0.098 (0.217)	0.512	0.127 (0.187)	0.113 (0.156)	0.881	0.315

[a] Data were presented as median (IQR)

[b] Statistical results were determined by using Wilcoxon matched-pairs signed rank test

[c] Statistical results were determined by using Mann Whitney test

Table 30. Comparison of pro- and anti-inflammatory cytokines pre- and post-intervention, and between SC and LcS groups using ELISA.

	SC (n=40) [a]			LcS (n=40) [a]			SC v.s. LcS (Post-test)
Parameter	Pre-test	Post-test	*p*-value [b]	Pre-test	Post-test	*p*-value [b]	*p*-value [c]
IL-6 (pg/mL)	11.34 (21.58)	7.06 (18.14)	0.678	5.12 (19.36)	7.70 (13.50)	0.916	0.923
IL-10 (pg/mL)	0.50 (2.95)	0.50 (2.83)	0.644	0.50 (8.32)	0.50 (4.66)	0.890	0.364
IL-17 (pg/mL)	9.38 (11.81)	9.38 (8.27)	0.946	9.38 (104.62)	9.38 (22.53)	0.491	0.068
TGF-β (ng/mL)	4.85 (13.77)	5.05 (12.12)	0.581	16.14 (17.96)	16.60 (17.10)	0.162	0.128
TNF-α (pg/mL)	20.09 (81.64)	29.30 (76.18)	0.072	7.71 (36.61)	7.94 (37.98)	0.034*	0.019*

[a] Data were presented as median (IQR)

[b] Statistical results were determined by using Wilcoxon matched-pairs signed rank test; *$p<0.05$

[c] Statistical results were determined by using Mann Whitney test; *$p<0.05$

Table 31. Comparison of antioxidant enzyme activity pre- and post-intervention, and between SC and LcS groups using the ELISA method.

	SC (n=40) [a]			LcS (n=40) [a]			SC v.s. LcS (Post-test)
Parameter	Pre-test	Post-test	*p*-value [b]	Pre-test	Post-test	*p*-value [b]	*p*-value [c]
CAT (U/mL)	33.81 (27.34)	35.38 (29.18)	0.942	27.55 (28.50)	26.16 (26.37)	0.454	0.028*
GPx (U)	103.30 (30.29)	105.80 (16.88)	0.805	89.86 (28.48)	105.20 (23.49)	0.016*	0.755
SOD (U/mL)	193.1 (140.2)	199.6 (100.6)	0.396	166.4 (107.6)	159.3 (118.9)	0.237	0.447

[a] Data were presented as median (IQR)

[b] Statistical results were determined by using Wilcoxon matched-pairs signed rank test; *$p<0.05$

[c] Statistical results were determined by using Mann Whitney test; *$p<0.05$

Table 32. Comparison of anabolic hormone levels pre- and post-intervention, and between SC and LcS groups using the ELISA method.

	SC (n=40) [a]			LcS (n=40) [a]			SC v.s. LcS (Post-test)
Parameter	Pre-test	Post-test	*p*-value [b]	Pre-test	Post-test	*p*-value [b]	*p*-value [c]
DHEA-S (μg/mL)	96.00 (61.80)	80.76 (70.70)	0.265	81.24 (66.43)	81.99 (70.19)	0.411	0.799

[a] Data were presented as median (IQR)

[b] Statistical results were determined by using Wilcoxon matched-pairs signed rank test

[c] Statistical results were determined by using Mann Whitney test

Table 33. Comparison of metal concentrations pre- and post-intervention, and between SC and LcS groups using ICP-MS.

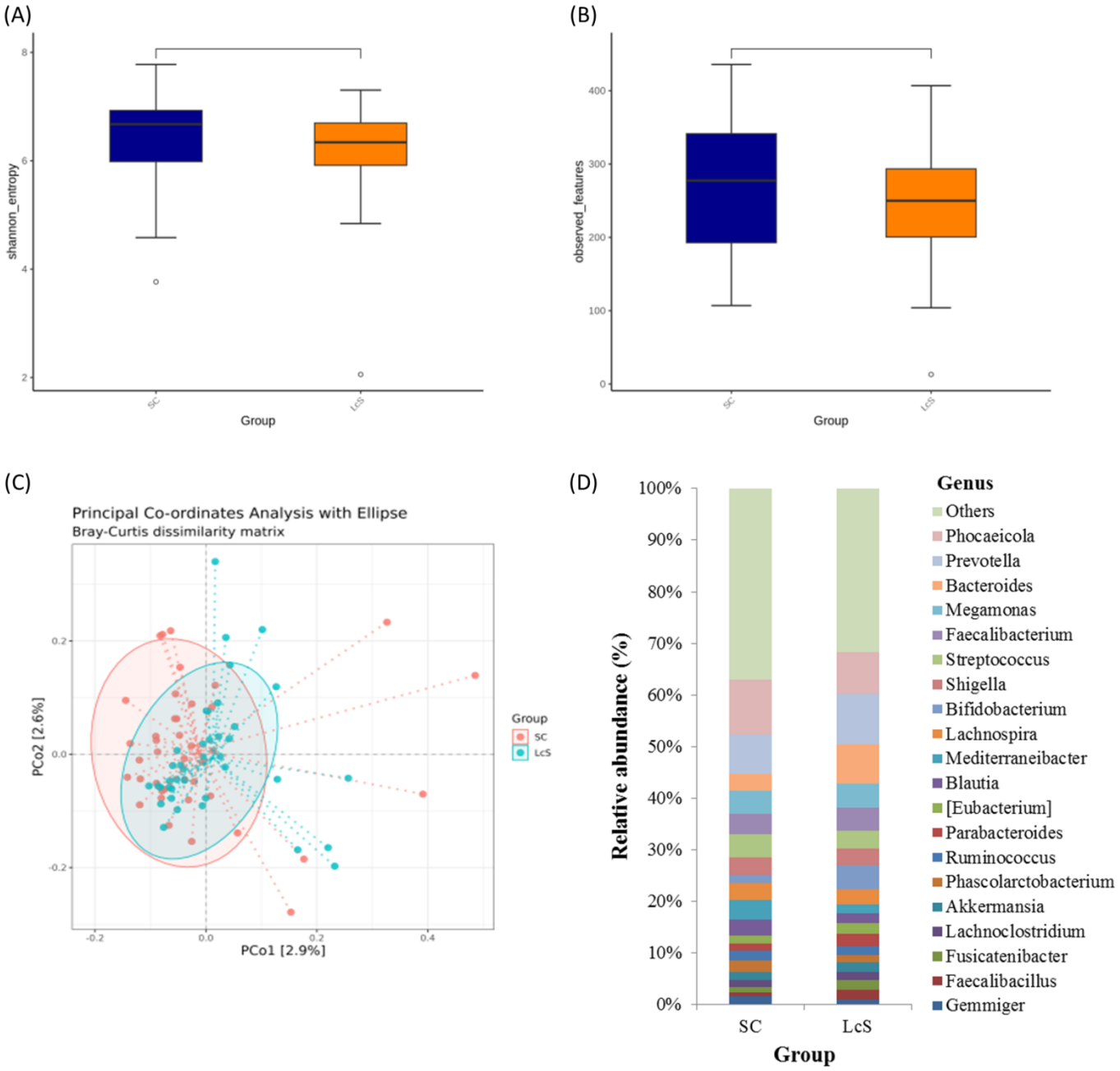
	SC (n=40) [a]			LcS (n=38) [a]			SC v.s. LcS (Post-test)
Metal (μg/L)	Pre-test	Post-test	*p*-value [b]	Pre-test	Post-test	*p*-value [b]	*p*-value [c]
Na	2664544 (1059721)	2739663 (622123)	0.685	2795566 (439564)	2322972 (664245)	0.000***	0.000***
Mg	42241 (9293)	42351 (13154)	0.627	43956 (7848)	36202 (13662)	0.000***	0.010*
K	2807697 (474408)	2852263 (752869)	0.476	2821855 (687371)	2262375 (799729)	0.000***	0.002**
Mn	10.80 (5.35)	11.83 (6.90)	0.349	11.56 (4.78)	9.47 (5.30)	0.002**	0.033*
Fe	398423 (82033)	387504 (79431)	0.314	397900 (70183)	327065 (137461)	0.000***	0.010*
Ni	1.92 (0.88)	1.62 (1.28)	0.020*	1.57 (0.97)	1.10 (0.92)	0.003**	0.026*
Cu	719.2 (121.1)	697.4 (162.8)	0.066	762.7 (117.6)	644.9 (167.6)	0.000***	0.039*
Zn	6298 (1427)	6105 (2070)	0.536	5869 (1199)	4691 (1903)	0.000***	0.011*
Sr	21.62 (9.93)	20.38 (11.50)	0.206	19.75 (11.00)	14.00 (6.17)	0.000***	0.000***
As	4.57 (5.67)	5.12 (4.76)	0.860	6.36 (6.79)	6.19 (6.13)	0.440	0.690
Se	363.7 (82.8)	381.6 (45.7)	0.189	368.9 (110.2)	355.3 (120.5)	0.447	0.183
Mo	0.98 (0.65)	1.14 (1.01)	0.131	0.98 (0.70)	1.01 (0.72)	0.691	0.128
Cd	0.62 (0.35)	0.66 (0.47)	0.261	0.56 (0.29)	0.52 (0.22)	0.272	0.015*
Sb	5.12 (2.53)	5.09 (2.36)	0.625	5.23 (3.37)	4.52 (2.12)	0.048*	0.020*
Ba	0.89 (0.58)	1.14 (1.44)	0.030*	0.90 (0.51)	0.64 (0.57)	0.027*	0.000***
Pb	14.14 (11.32)	15.43 (14.50)	0.722	13.61 (9.80)	10.03 (6.62)	0.000***	0.004**

[a] Data were presented as median (IQR)

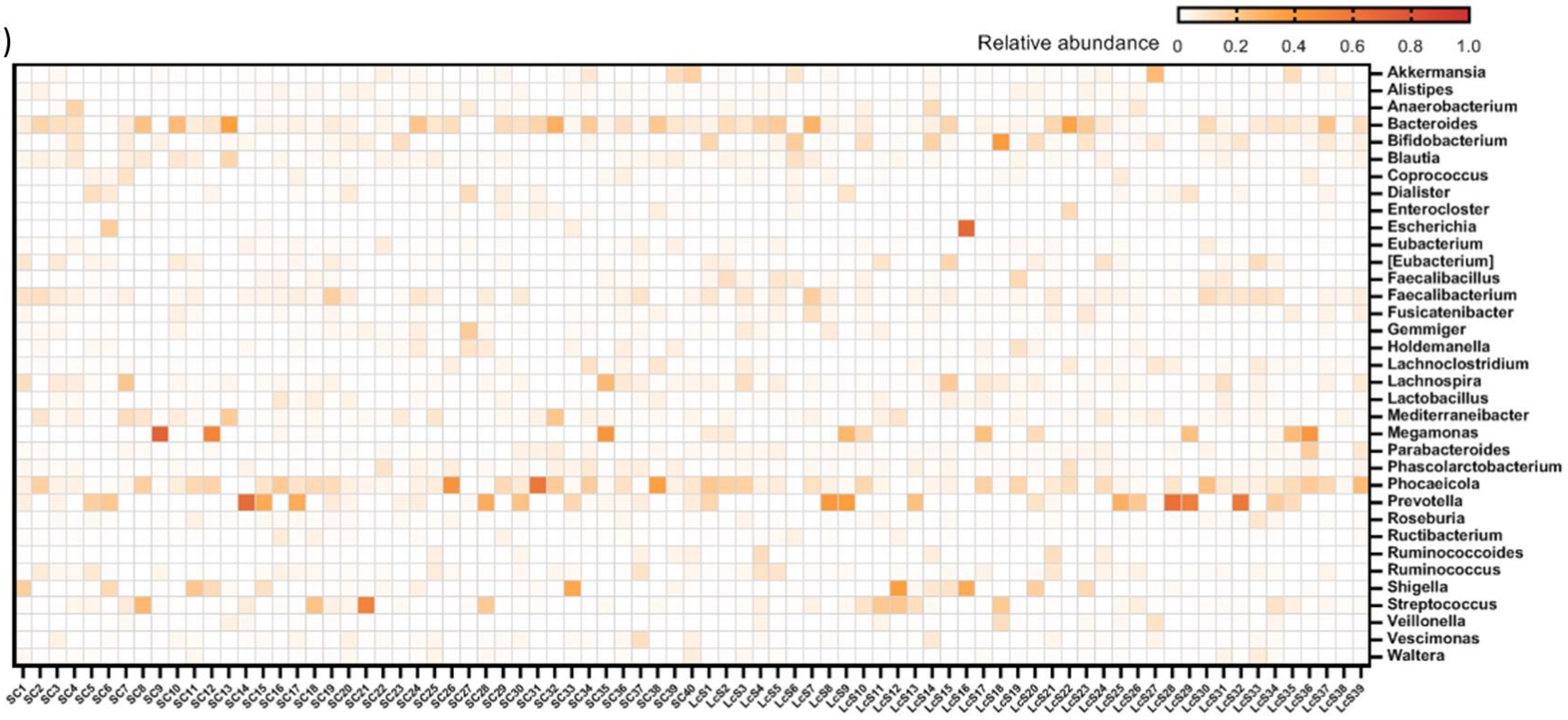
[b] Statistical results were determined by using Wilcoxon matched-pairs signed rank test; *p<0.05, **p<0.01, ***p<0.001

[c] Statistical results were determined by using Mann Whitney test; *p<0.05, **p<0.01, ***p<0.001

Gut microbiota composition and microbiota-derived Metabolite Analysis:



(E)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0.4
0.6
0.8
1.0
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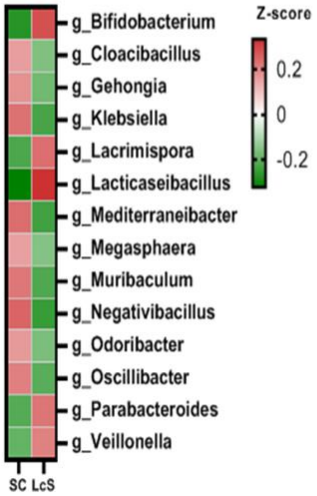
Alistipes
Anaerobacterium
Bacteroides
Bifidobacterium
Blautia
Coprococcus
Dialister
Enterocloster
Escherichia
Eubacterium
[Eubacterium]
Faecalibacillus
Faecalibacterium
Fusicatenibacter
Gemmiger
Holdemanella
Lachnoclostridium
Lachnospira
Lactobacillus
Mediterraneibacter
Megamonas
Parabacteroides
Phascolarctobacterium
Phocaeicola
Prevotella
Roseburia
Ructibacterium
Ruminococcoides
Ruminococcus
Shigella
Streptococcus
Veillonella
Vescimonas
Waltera

(F)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g_Klebsiella
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g_Lacticaseibacillus
g_Mediterraneibacter
g_Megasphaera
g_Muribaculum
g_Negativibacillus
g_Odoribacter
g_Oscillibacter
g_Parabacteroides
g_Veillonella
SC
LcS
Z-score
0.2
0
-0.2

Figure 5. Alterations of gut microbiota composition between Sarcopenia Control (SC) and Sarcopenia + Lacticaseibacillus casei Shirota (LcS) groups.

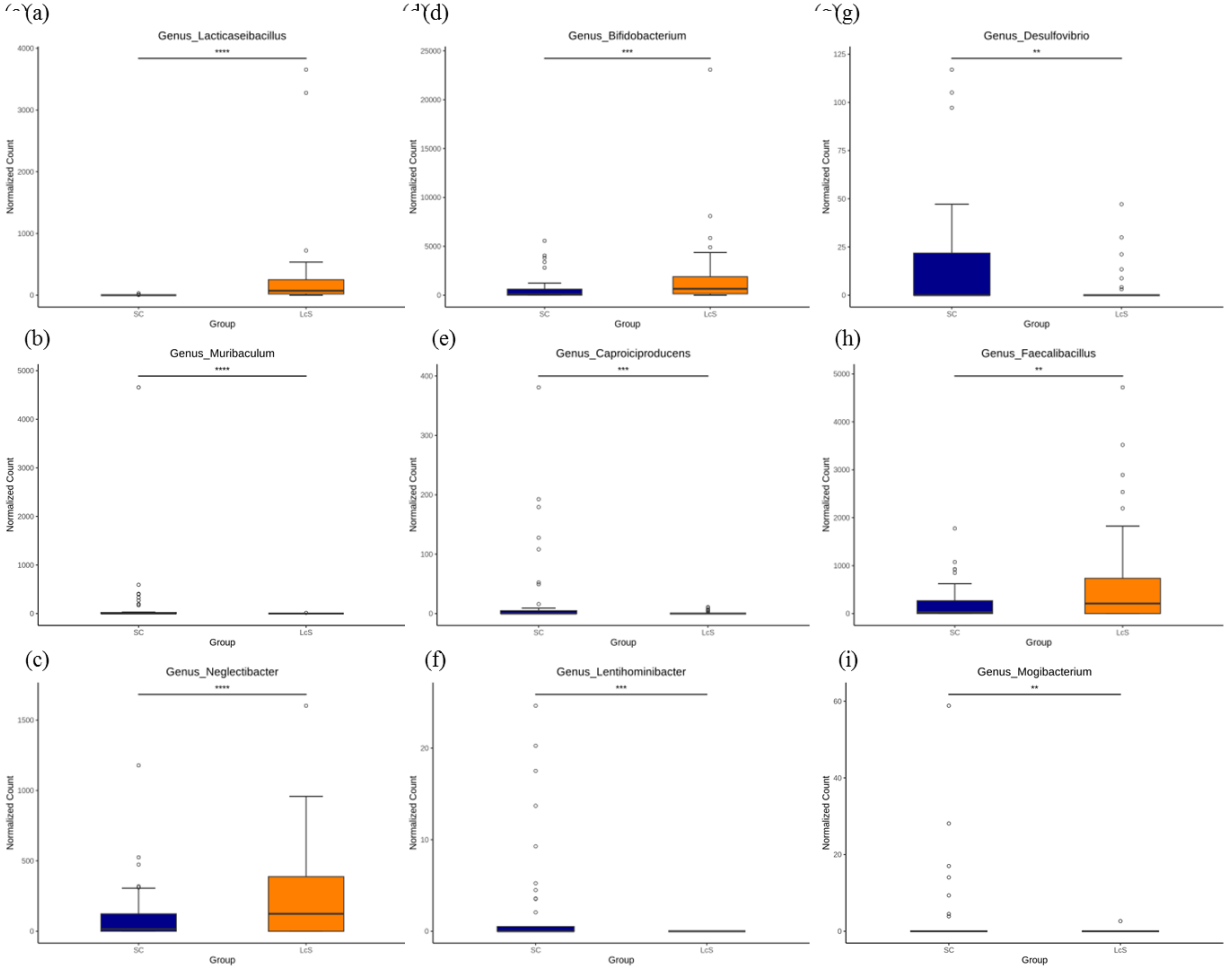
(A & B) Box-and-whisker plots show α-diversity indices (Shannon index and Observed features index) comparing SC and LcS groups.

(C) β-diversity assessed using Bray-Curtis distance and visualized by PCoA.

(D) Bar plot showing the relative abundance of the top 20 bacterial genera.

(E) Heatmap showing the relative abundance of the top 35 gut microbiota genera across individual subjects. Each vertical column represents one subject, and the color intensity indicates the abundance level. Red denotes higher relative abundance.

(F) Heatmap showing taxa with significant linear discriminant analysis (LDA) scores (>3.0), indicating genera that are differentially abundant between SC and LcS, as identified by LEfSe. The heatmap displays Z-score–normalized relative abundance values. For each genus, the relative abundance was averaged within each group and then standardized using Z-score transformation across groups. Deep red indicates higher relative abundance; deep green indicates lower abundance. Only genus-level taxa are shown, and they are presented in alphabetical order for clarity and consistency with the main text.


(a)
Genus_Lacticaseibacillus
(d)
Genus_Bifidobacterium
(g)
Genus_Desulfovibrio
(b)
Genus_Muribaculum
(e)
Genus_Caproiciproducens
(h)
Genus_Faecalibacillus
(c)
Genus_Neglectibacter
(f)
Genus_Lentihominibacter
(i)
Genus_Mogibacterium
Normalized Count
Group
SC
LcS

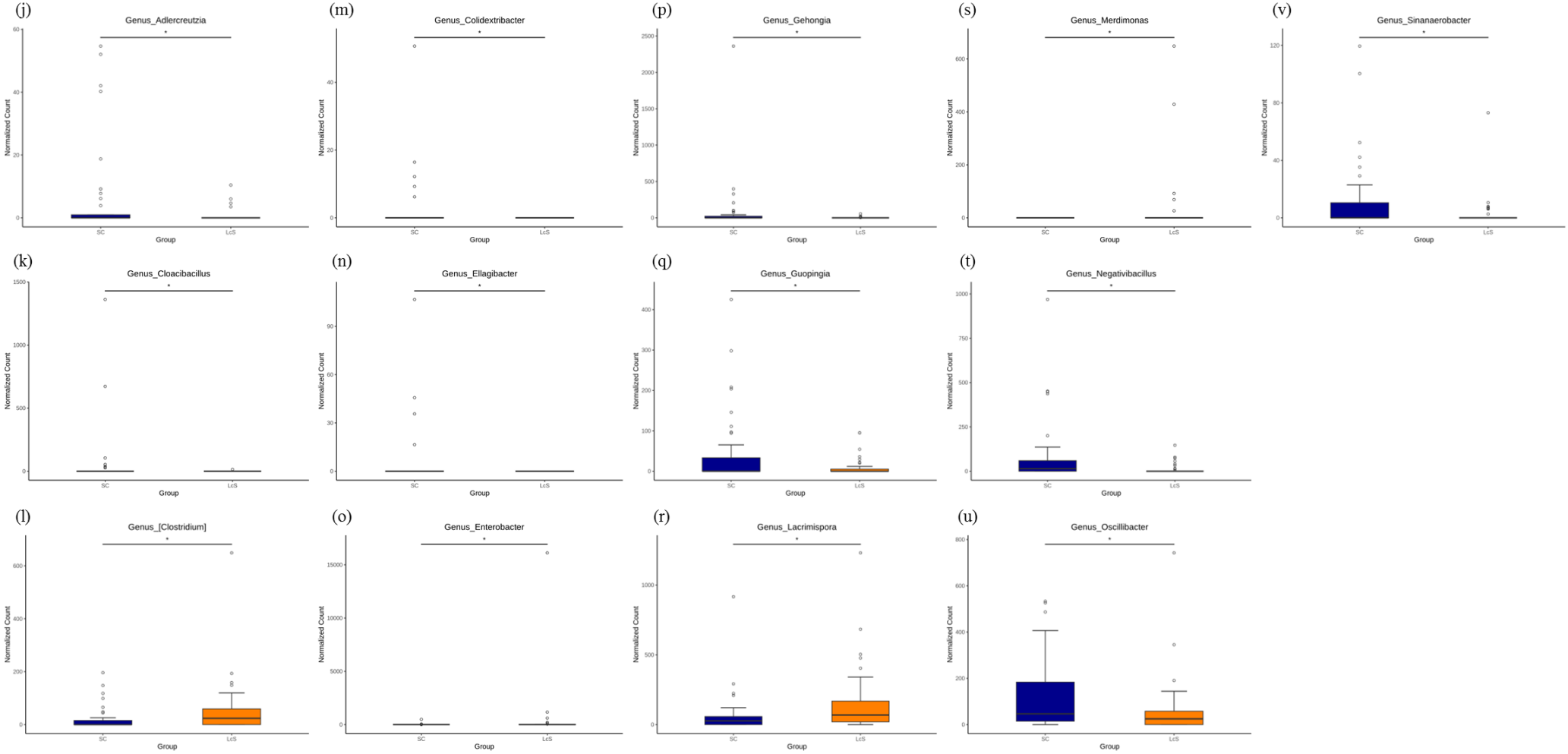


Figure 6. MetagenomeSeq analysis of gut microbiota compared SC group and LcS group.

Bacterial genus that were significantly more abundant between SC group and LcS group with high adherence (FDR < 0.05) by metagenomeSeq test, including (a)*Lacticaseibacillus*, (b)*Muribaculum*, (c)*Neglectibacter*, (d)*Bifidobacterium*, (e)*Caproiciproducens*, (f)*Lentihominibacter*, (g)*Desulfovibrio*,

(h)*Faecalibacillus*, (i)*Mogibacterium*, (j)*Adlercreutzia*, (k)*Cloacibacillus*, (l)*[Clostridium]*, (m)*Colidextribacter*, (n)*Ellagibacter*, (o)*Enterobacter*, (p)*Gehongia*, (q)*Guopingia*, (r)*Lacrimispora*, (s)*Merdimonas*, (t)*Negativibacillus*, (u)*Oscillibacter*, and (v)*Sinanaerobacter*.

Table 34. Differentially Abundant Genera Between LcS and SC Identified by LEfSe and MetagenomeSeq

Category	Increased	Decreased
(1) Both LEfSe and MetagenomeSeq	*Bifidobacterium, Lacrimispora, Lacticaseibacillus,*	*Cloacibacillus, Gehongia, Muribaculum, Negativibacillus, Oscillibacter*
(2) LEfSe only	*Megamonas, Streptococcus,*	*Klebsiella, Mediterraneibacter, Megasphaera, Odoribacter*
(3) MetagenomeSeq only	*[Clostridium], Enterobacter, Faecalibacillus, Merdimonas, Neglectibacter*	*Adlercreutzia, Caproiciproducens, Colidextribacter, Desulfovibrio, Ellagibacter, Guopingia, Lentihominibacter, Mogibacterium, Sinanaerobacter*

The direction of change is indicated relative to the SC group, with genera classified as significantly increased or decreased in the LcS group. Results are categorized based on detection by (1) both LEfSe and MetagenomeSeq, (2) LEfSe only, or (3) MetagenomeSeq only.

Table 35 . Comparison of metabolite analysis among NS, SC, and LcS groups using LC-MS/MS.

Group	NS	SC	LcS	Kruskal-Wallis test	NS vs SC	NS vs LcS	SC vs LcS
	median (IQR)				p-value [a]		
Acetic acid (nM)	2,038.82 (1,561.23)	1,357.79 (1,147.08)	1,635.15 (824.05)	0.0025**	**	ns	ns
Propionic acid (nM)	1,789.35 (1,467.82)	1,582.41 (1,418.59)	1,711.42 (1,091.85)	0.289	ns	ns	ns
Butyric acid (nM)	698.14 (769.52)	468.83 (509.72)	450.01 (637.81)	0.0433	ns	ns	ns
Isobutyric acid (nM)	101.89 (72.15)	100.83 (56.38)	83.58 (63.58)	0.3817	ns	ns	ns
Valeric acid (nM)	161.66 (141.77)	161.87 (169.79)	128.13 (135.24)	0.22	ns	ns	ns
Isovaleric acid (nM)	61.46 (58.32)	199.85 (139.80)	88.11 (90.96)	<0.0001***	***	ns	***
TMAO (nM)	1.02 (0.23)	1.07 (1.20)	1.06 (0.49)	0.824	ns	ns	ns
IPA (nM)	361.75 (292.59)	372.62 (264.91)	479.96 (463.63)	0.378	ns	ns	ns

[a] Statistical results were determined by using Kruskal-Wallis test and Dunn's Multiple Comparison Test; ns: non-significant, *p<0.05, **p<0.01, ***p<0.001

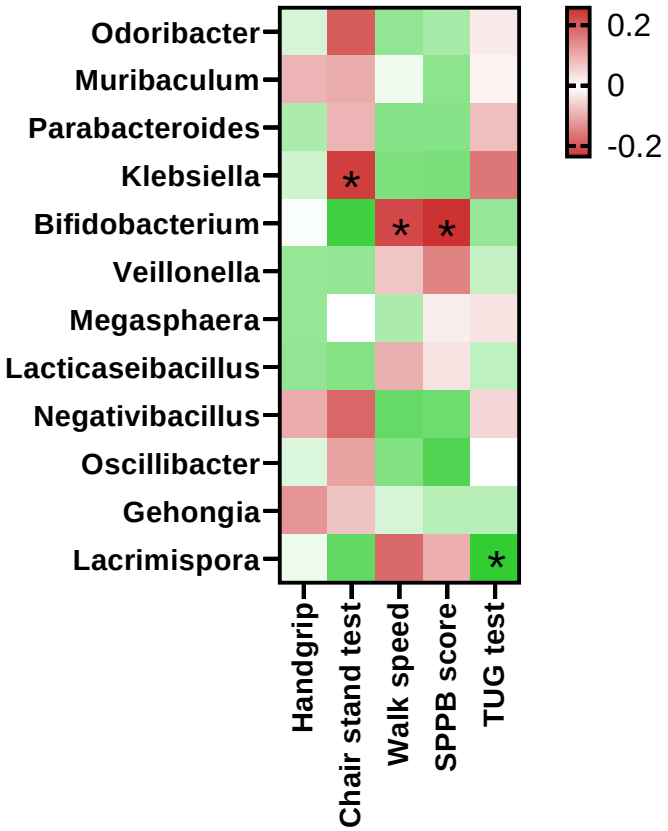


Figure 7. The spearman correlations between gut microbiota and physical examinations

The heat map shows the Spearman correlations between gut microbiota and physical examinations in SC (n=40) and LcS (n=40) subjects; *p<0.05

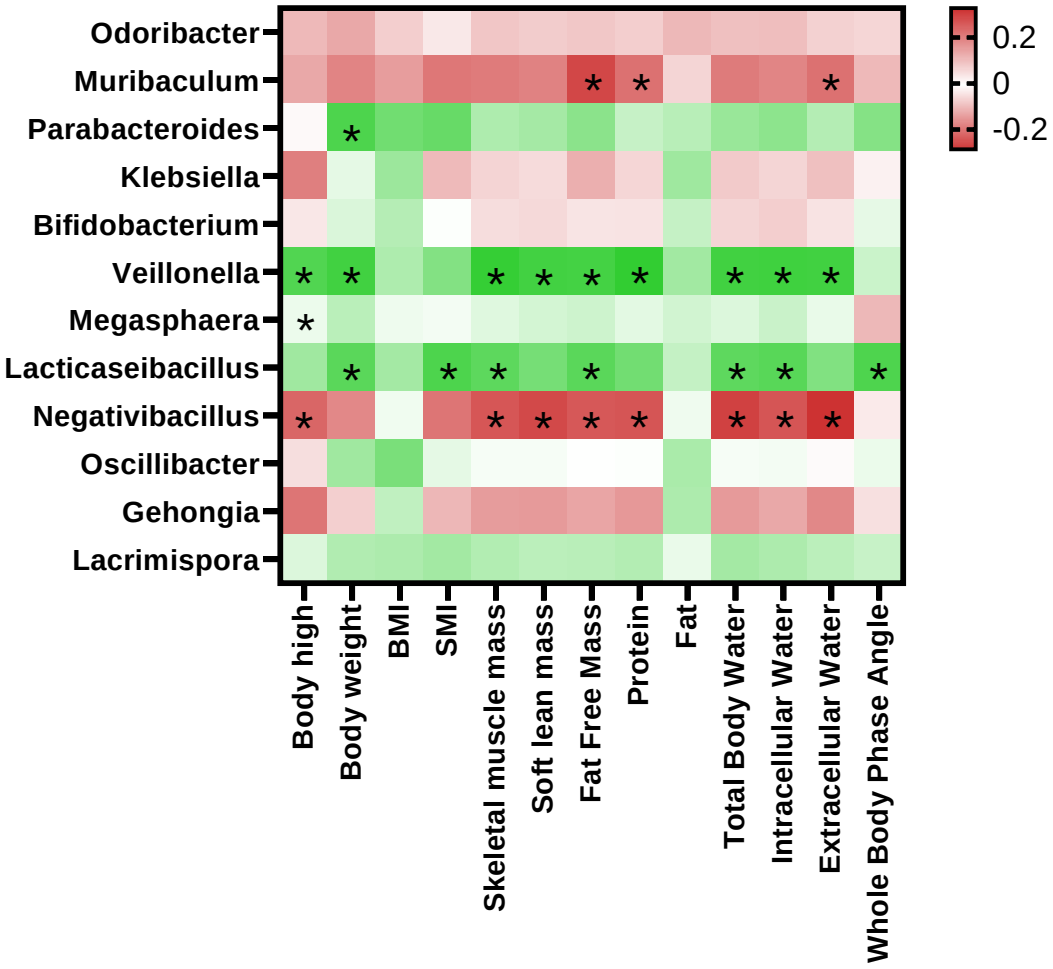


Figure 8. the Spearman correlations between gut microbiota and body compositions

The heat map displayed the Spearman correlations between gut microbiota and body compositions in SC (n=40), and LcS (n=40) subjects; *p<0.05.

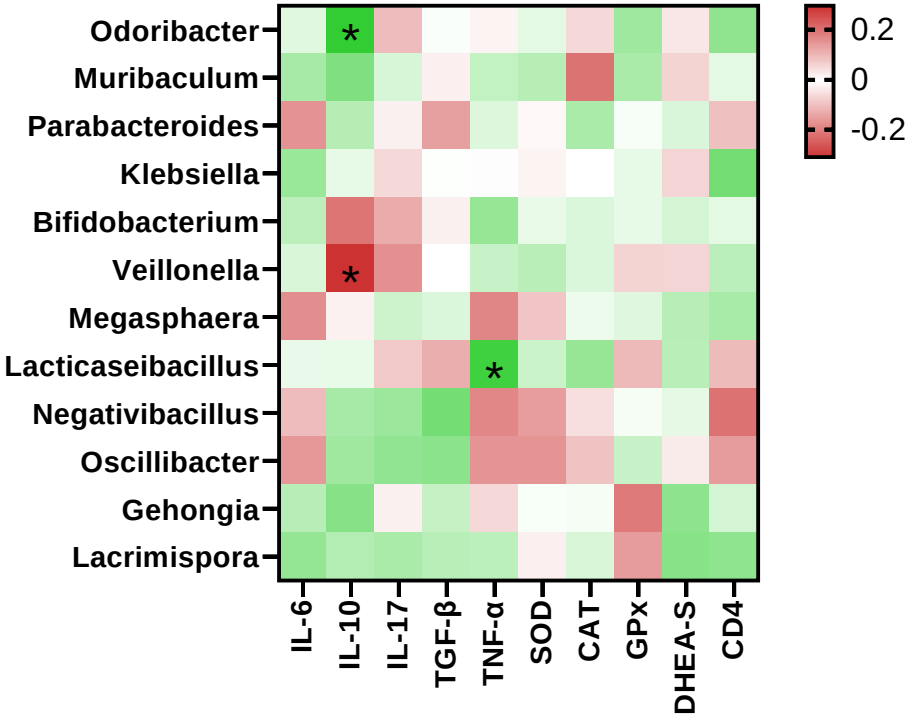


Figure 9. The Spearman correlations between gut microbiota and cytokines, DHEA-S and CD4 lymphocyte

The heat map displayed the Spearman correlations between gut microbiota and cytokines, DHEA-S and CD4 lymphocyte in SC (n=40), and LcS (n=40) subjects; *p<0.05.

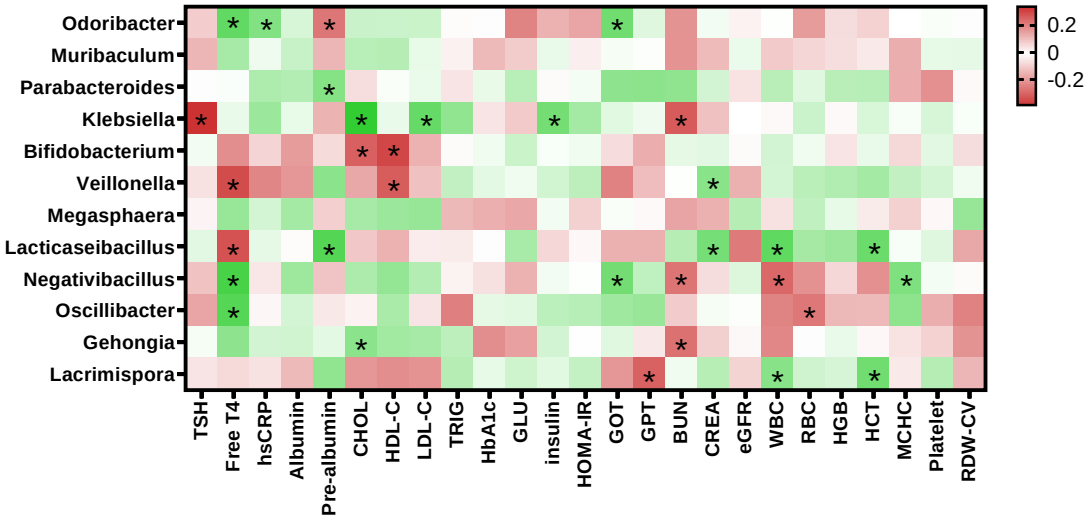


Figure 10. The Spearman correlations between gut microbiota and biochemical parameters

The heat map displayed the Spearman correlations between gut microbiota and biochemical parameters in SC (n=40), and LcS (n=40) subjects; *p<0.05.

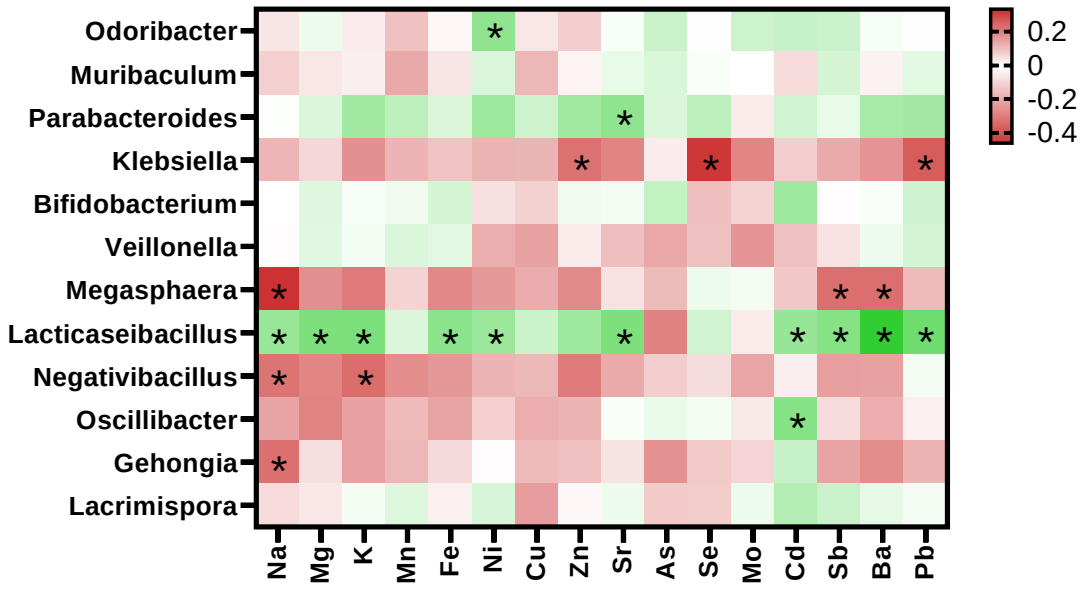


Figure 11. The Spearman correlations between gut microbiota and heavy metals

The heat map displayed the Spearman correlations between gut microbiota and heavy metals in SC (n=40), and LcS (n=40) subjects; *p<0.05.

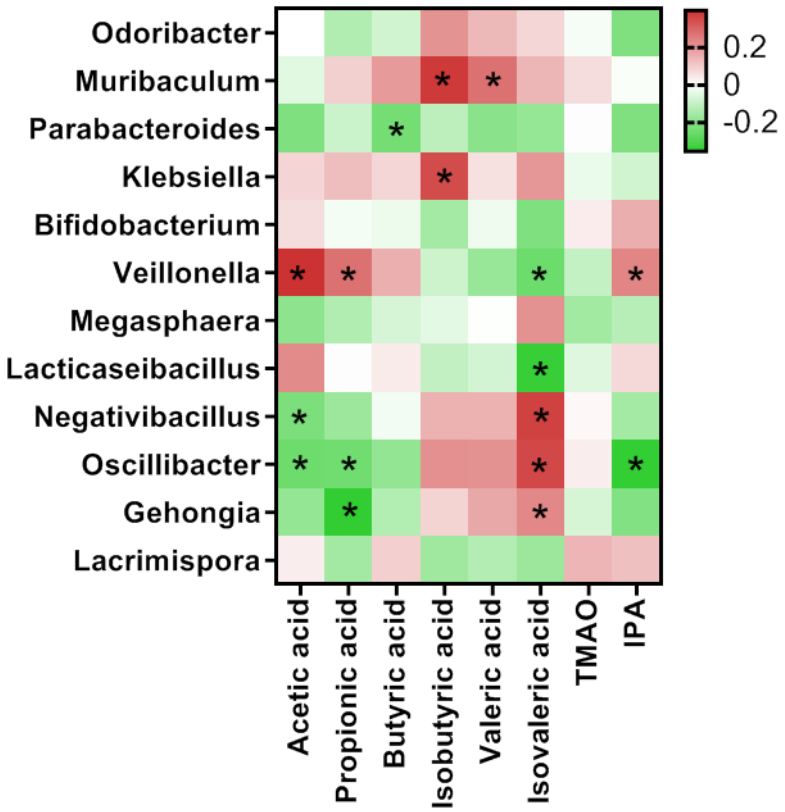
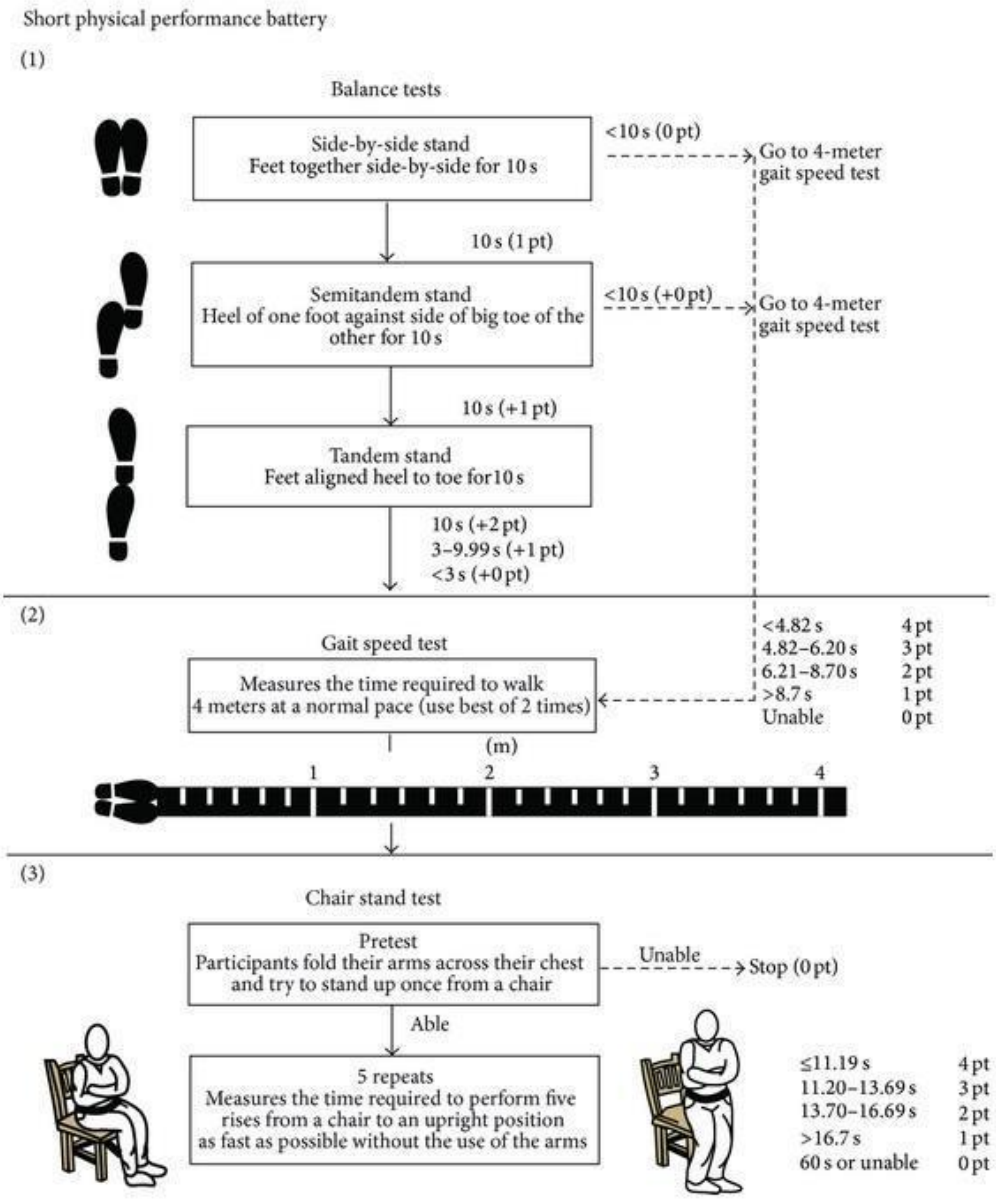


Figure 12. The Spearman correlations between gut microbiota and metabolites

The heat map displayed the Spearman correlations between gut microbiota and metabolites in SC (n=40), and LcS (n=40) subjects; *p<0.05.

Appendix

Appendix I



Appendix II

Basic information		
Name：	**Number：**	**Date：**
Personal history		
Smoking	☐No ☐Quit for _____ years ☐Yes, Average daily _____ packages	
Drinking	☐No ☐ Quit for _____ years ☐ occasionally ☐ Drink often	
Chewing betel nut	☐No ☐Quit for _____ years ☐ occasionally ☐ often	
Food allergy	☐No ☐Yes_____	
Past medical history	☐Cerebrovascular disease ☐Chronic obstructive pulmonary disease ☐ congestive heart failure ☐ hypertension ☐ Hyperlipidemia ☐ Dementia ☐ Arrhythmia ☐ Diabetes mellitus type 2 ☐ Diabetes with kidney disease, eye, and nerve ☐ Paralysis of lower body ☐ Cancer ☐ metastatic carcinoma ☐ Cirrhosis, biliary cirrhosis, chronic hepatitis ☐ Moderate to severe liver disease☐ Acute myocardial infarction ☐ acute perforation of gastro-duodenal ulcer ☐ peipheral vscular disease ☐ kidney disease ☐ Rheumatology and Immunology disease ☐other_____	

Appendix Ⅲ

24 hours dietary recall

Name：_____ Date：_____

Meals	Time	Place	Food name	Serving size (please estimate by bowl or spoon)	Preparation (Fried, fried, fried, steamed, (Boil, stew, stew, roast)	Remarks
Breakfast						
Lunch						
Dinner						
Dessert						

Appendix IV

- We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the last 7 days. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.
- Think about all the vigorous activities that you did in the last 7 days. Vigorous physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

1. During the last 7 days, on how many days did you do vigorous physical activities like heavy lifting, digging, aerobics, or fast bicycling?
_____ days per week
No vigorous physical activities Skip to question 3

2. How much time did you usually spend doing vigorous physical activities on one of those days?
_____ hours per day
_____ minutes per day
Don't know/Not sure

Think about all the moderate activities that you did in the last 7 days. Moderate activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

3. During the last 7 days, on how many days did you do moderate physical activities like carrying light loads, bicycling at a regular pace, or doubles tennis?
Do not include walking.
_____ days per week
No moderate physical activities Skip to question 5

4. How much time did you usually spend doing moderate physical activities on one of those days?
_____ hours per day
_____ minutes per day
Don't know/Not sure
Think about the time you spent walking in the last 7 days. This includes at work and at home, walking to travel from place to place, and any other walking that you have done solely for recreation, sport, exercise, or leisure.

5. During the last 7 days, on how many days did you walk for at least 10 minutes at a time?
_____ days per week
No walking Skip to question 7

6. How much time did you usually spend walking on one of those days?
_____ hours per day
_____ minutes per day
Don't know/Not sure

The last question is about the time you spent sitting on weekdays during the last 7 days. Include time spent at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading, or sitting or lying down to watch television.

7. During the last 7 days, how much time did you spend sitting on a week day?
_____ hours per day
_____ minutes per day
Don't know/Not sure

This is the end of the questionnaire, thank you for participating.

Appendix V

1. How often do you consume fish (including all sort of fish)? (Silver pomfret, seabass, crucian carp, snapper, tilapia, various kinds of canned fish, etc.)
   (1) Every day, _____ time(s) a day.
   (2) Every week, _____ time(s) a week.
   (3) Every month, _____ time(s) a month.
   (4) Never

2. Did you consume any deep sea fish (high ω-3 fatty acids contained) one month ago? (Cod, spearfish, shark, mackerel, salmon, tuna, canned deep-sea fish, etc.)
   (1) Every day, _____ time(s) a day.
   (2) Every week, _____ time(s) a week.
   (3) Every month, _____ time(s) a month.
   (4) Never

3. Did you consume any aquatic fish with bone one month ago? (Dried shrimp, shell shrimp, soft crab, larva fish, dried fish, canned fish with bone, etc.)
   (1) Every day, _____ time(s) a day.
   (2) Every week, _____ time(s) a week.
   (3) Every month, _____ time(s) a month.
   (4) Never

4. Did you consume any shells one month ago? (Oyster, dried oyster, clams, corbicula, spiral shell, ear shell, scallop, Taiwan abalone, etc.)
   (1) Every day, _____ time(s) a day.
   (2) Every week, _____ time(s) a week.
   (3) Every month, _____ time(s) a month.
   (4) Never

5. Did you consume any other kind of seafood except shells one month ago? (Shrimp, neritic squid, common squid, cuttlefishes, octopus, inkfish, clams, serrated crab, trepang, etc.)
   (1) Every day, _____ time(s) a day.
   (2) Every week, _____ time(s) a week.
   (3) Every month, _____ time(s) a month.
   (4) Never

6. Did you consume any seafood with roe one month ago? (Fish roe, mullet roe, etc.)
   (1) Every day, _____ time(s) a day.
   (2) Every week, _____ time(s) a week.
   (3) Every month, _____ time(s) a month.
   (4) Never

7. Did you consume any seafood products one month ago? (Shredded fish, mullet roe, fish cake, salted fish, squid shreds, katsuobushi, etc.)
   (1) Every day, _____ time(s) a day.
   (2) Every week, _____ time(s) a week.
   (3) Every month, _____ time(s) a month.
   (4) Never

8. Did you consume any poultry one month ago? (Chicken, duck, goose, etc.)
   (1) Every day, _____ time(s) a day.
   (2) Every week, _____ time(s) a week.
   (3) Every month, _____ time(s) a month.
   (4) Never

9. Did you consume any livestock one month ago? (Pork, beef, lamb, etc.)
   (1) Every day, _____ time(s) a day.
   (2) Every week, _____ time(s) a week.
   (3) Every month, _____ time(s) a month.
   (4) Never

10. Did you consume any livestock of fatty meat one month ago? (Hock, pigs feet, ground meat, marbled beef , hamburg, bao, dumpling's stuffing, etc.)
   (1) Every day, _____ time(s) a day.
   (2) Every week, _____ time(s) a week.
   (3) Every month, _____ time(s) a month.
   (4) Never

11. Did you consume any liver one month ago? (Pork, chicken's, beef's, duck's and goose's liver, etc.)
   (1) Every day, _____ time(s) a day.
   (2) Every week, _____ time(s) a week.
   (3) Every month, _____ time(s) a month.
   (4) Never

12. Did you consume other kinds of organs except liver one month ago? (Pig's small intestines, pig bag, pig's heart, chitterlings, chicken's heart, gizzards, kidney, etc.)
    (1) Every day, _____ time(s) a day.
    (2) Every week, _____ time(s) a week.
    (3) Every month, _____ time(s) a month.
    (4) Never

13. Did you consume any poultry's or livestock's blood one month ago? (Black pudding, duck blood, etc.)
    (1) Every day, _____ time(s) a day.
    (2) Every week, _____ time(s) a week.
    (3) Every month, _____ time(s) a month.
    (4) Never

14. Did you consume any meat products one month ago? (Dried meat, meat floss, sausage, ham, bacon, cured meat, smoked and corned duck meet, hot dog, etc.)
    (1) Every day, _____ time(s) a day.
    (2) Every week, _____ time(s) a week.
    (3) Every month, _____ time(s) a month.
    (4) Never

15. Did you consume any meat products one month ago? (Fish ball, pork ball, beef ball, cuttlefish ball, bean curd skin, tempura, pork thick soup, squid thick soup, etc.)
    (1) Every day, _____ time(s) a day.
    (2) Every week, _____ time(s) a week.
    (3) Every month, _____ time(s) a month.
    (4) Never

16. Did you consume any gluten or rolled flour gluten one month ago? (Gluten with peanuts, rolled flour gluten, etc.)
    (1) Every day, _____ time(s) a day.
    (2) Every week, _____ time(s) a week.
    (3) Every month, _____ time(s) a month.
    (4) Never

17. Did you consume any soy bean products one month ago? (Tofu, oily tofu, skin of bean curd, red jujube bean, hundred layered bean curd, dried tofu, vegetarian chicken, vegetarian ham, etc.)
    (1) Every day, _____ time(s) a day.
    (2) Every week, _____ time(s) a week.
    (3) Every month, _____ time(s) a month.
    (4) Never

18. Did you consume any soy bean fermented products one month ago? (Soy cheese, stinky tofu, miso, etc.)
    (1) Every day, _____ time(s) a day.
    (2) Every week, _____ time(s) a week.
    (3) Every month, _____ time(s) a month.
    (4) Never

19. Did you consume any soy milk, soybean milk, or soybean pudding one month ago?
    (1) Every day, _____ time(s) a day.
    (2) Every week, _____ time(s) a week.
    (3) Every month, _____ time(s) a month.
    (4) Never

20. The following options are about the soy milk products; please choose soy milk products you consume frequently:
    (1) Soy milk (2) Fresh milk soy milk (3) Soy milk with high calcium
    (4) Lecithin soy milk (5) Peanut rice milk (6) Black soybean milk
    (7) Honey soy milk (8) Soybean pudding (9) Other
    (10) None

21. Did you consume any green pea, legume one month ago? (Wax bean, string bean, kidney bean, green peas, pease, pea, green soybean, lima bean, etc.)
    (1) Every day, _____ time(s) a day.
    (2) Every week, _____ time(s) a week.
    (3) Every month, _____ time(s) a month.
    (4) Never

Appendix VI

Basic information

1. ( ) What is the highest degree or level of education you have completed?
   (0) Illiteracy (1) Elementary school (2) Junior high school (3) Senior high school (4) Five-year junior college program (5) Two-year technical program (6) College or university (7) Graduate School.
2. ( ) What is your total annual household income?
   (0) Less than NT$500000 (1) NT$500000- NT$999999 (2) NT$1000000~ NT$1499999(3) NT$1500000~ NT$1999999 (4) 2 million NT dollars or more.

Exposure assessment

3. ( ) Do you feel exposure to cooking oil fume?
   (0) No **(continue with Question 4)** (1) Yes **(continue with Question 3.1)**
   3.1( ) Fume extractor use?
   (0) No (1) Yes, sometimes (≦3times/week) (2) Yes, always (>3times/week).
4. ( ) Do you use incense at home?
   (0)No (1)Yes, ＜1 times/month (2)Yes, ≧1 times/month ~＜1 times/week
   (3)Yes, ≧1 times/week ~＜1 times/day (4) Yes, ≧1 times/day.
5. ( ) Do you use mosquito-repelling incense?
   (0)No (1)Yes, ＜1 times/month (2)Yes, ≧1 times/month ~＜1 times/week
   (3)Yes, ≧1 times/week ~＜1 times/day (4) Yes, ≧1 times/day.

Dietary habits

6. ( ) Do you have the habit of smoking?
   (smoking less than 1 cigarette/day or less than 20 packs in your life, answer No)
   (0) No **(continue with Question 7)** (1) Yes **(continue with Question 6.1).**
   6.1 How many years total have you smoked cigarettes?_____Years, _____cigarettes/day?
7. ( ) Does passive smoking around you ? (0) No (1) Yes.
8. ( ) Do you have the habit of drinking? **(over than one time/week, answer YES)**
   (0) No **(continue with Question 9)** (1)Yes **(continue with Question 8.1).**
   8.1How may years for drinking? _____Years, _____times/week, average volume _____ c.c
   (cans 330 c.c., glass bottle 600 c.c., glass cup 150 c.c.)
   8.2 ( ) Have you ever quit drinking?
   (0) No (1) Yes, I quit drinking (2) Yes, but it hasn't worked very long.
9. ( ) Do you have the habit of drinking coffee? **(more than one time/week, answer YES)**
   (0) No **(continue with Question 10)** (1) Yes **(continue with Question 9.1)**
   9.1 Average volume per week_____ c.c.
   (7-11 city café Large: 16oz≒480c.c.; median 12oz≒360 c.c.; small 8oz≒240 c.c.)
10. ( ) Do you have the habit of drinking tea? **(more than one time/week, answer YES)**
    (0) No **(continue with Question 11)** (1) Yes **(continue with Question 10.1)**
    10.1 Average volume per week_____ c.c. **(bottled ≒600 c.c., Hand-shake tea≒700 c.c.)**
11. ( ) Do you have the habit of chewing of betel nut? **(more than a betel nut/week, answer YES)**
    (0) No **(continue with Question 12)** (1) Yes **(continue with Question 11.1)**
    11.1How many years for chewing betel nut_____Years, _____pieces.
    11.2( ) Have you ever quit betel nut? (0) No (1)Yes, I quit (2) Yes, but it hasn't worked very long.
12. ( ) Do you have the habit of taking a supplement in nearly one month?
    (0) No **(continue with Question 13)** (1) Yes **(continue with Question 12.1)**
    12.1Supplement brand:
    12.2 ( ) How often you take the supplements? (0)1~3 pieces/month (1) 4~6 pieces/week(2) 1piece/day (3) over than 1piece/day.
13. ( ) Do you have the habit of seafood consumption? **(more than one time/week, answer YES)**
    (0) No (1) Yes **(continue with Question 13.1)**
    13.1 How often you consumption the seafood _____ per week?

Appendix Ⅶ

Patient Assessment of Constipation symptom (PAC-SYM)						
Symptom		Level of severity(Likert 5 point scale)				
		Absent	Mild	Moderate	Severe	Very severe
		0	1	2	3	4
Feces	Hard bowel movements					
Rectal symptom	Small bowel movements					
	Straining bowel movements					
	Painful bowel movements					
	Incompelete bowel movements					
	False alarms					
	Rectal bleeding					
	Rectal burning					
Abdomen symptom	Stomach cramps					
	Pain in abdomen					
	Bloating in abdomen					
	Discomfort in abdomen					
Score						

Appendix VIII

SARC-F

Components	Questions	SARC-F score	score
Strength	How much difficulty do you have in lifting and carrying 10 pounds?	None=0 Some = 1 A lot or unable = 2	
Assistance in walking	How much difficulty do you have walking across a room?	None = 0 Some = 1 A lot, use aids, or unable = 2	
Rise from a chair	How much difficulty do you have transferring from a chair or bed?	None = 0 Some = 1 A lot or unable without help = 2	
Clime stairs	How much difficulty do you have climbing a flight of 10 stairs?	None = 0 Some = 1 A lot or unable = 2	
Falls	How many times have you fallen in the past year?	None = 0 1–3 falls = 1 4 or more falls = 2	

Appendix Ⅸ

MINI NUTRITIONAL ASSESSMENT
MNA®

ID# _____

Last Name: _____ First Name: _____ M.I. _____ Sex: _____ Date: _____

Age: _____ Weight, kg: _____ Height, cm: _____ Knee Height, cm: _____

Complete the form by writing the numbers in the boxes. Add the numbers in the boxes and compare the total assessment to the Malnutrition Indicator Score.

ANTHROPOMETRIC ASSESSMENT

	Points
1. Body Mass Index (BMI) (weight in kg) / (height in m)² a. BMI < 19 = 0 points b. BMI 19 to < 21 = 1 point c. BMI 21 to < 23 = 2 points d. BMI ≥ 23 = 3 points	☐
2. Mid-arm circumference (MAC) in cm a. MAC < 21 = 0.0 points b. MAC 21 ≤ 22 = 0.5 points c. MAC > 22 = 1.0 points	☐.☐
3. Calf circumference (CC) in cm a. CC < 31 = 0 points b. CC ≥ 31 = 1 point	☐
4. Weight loss during last 3 months a. weight loss greater than 3kg (6.6 lbs) = 0 points b. does not know = 1 point c. weight loss between 1 and 3 kg (2.2 and 6.6 lbs) = 2 points d. no weight loss = 3 points	☐

GENERAL ASSESSMENT

5. Lives independently (not in a nursing home or hospital) a. no = 0 points b. yes = 1 point	☐
6. Takes more than 3 prescription drugs per day a. yes = 0 points b. no = 1 point	☐
7. Has suffered psychological stress or acute disease in the past 3 months a. yes = 0 points b. no = 2 points	☐
8. Mobility a. bed or chair bound = 0 points b. able to get out of bed/chair but does not go out = 1 point c. goes out = 2 points	☐
9. Neuropsychological problems a. severe dementia or depression = 0 points b. mild dementia = 1 point c. no psychological problems = 2 points	☐
10. Pressure sores or skin ulcers a. yes = 0 points b. no = 1 point	☐

DIETARY ASSESSMENT

11. How many full meals does the patient eat daily? a. 1 meal = 0 points b. 2 meals = 1 point c. 3 meals = 2 points	☐

	Points
12. Selected consumption markers for protein intake • At least one serving of dairy products (milk, cheese, yogurt) per day? yes ☐ no ☐ • Two or more servings of legumes or eggs per week? yes ☐ no ☐ • Meat, fish or poultry every day? yes ☐ no ☐ a. if 0 or 1 yes = 0.0 points b. if 2 yes = 0.5 points c. if 3 yes = 1.0 points	☐.☐
13. Consumes two or more servings of fruits or vegetables per day? a. no = 0 points b. yes = 1 point	☐
14. Has food intake declined over the past three months due to loss of appetite, digestive problems, chewing or swallowing difficulties? a. severe loss of appetite = 0 points b. moderate loss of appetite = 1 point c. no loss of appetite = 2 points	☐
15. How much fluid (water, juice, coffee, tea, milk,...) is consumed per day? (1 cup = 8 oz.) a. less than 3 cups = 0.0 points b. 3 to 5 cups = 0.5 points c. more than 5 cups = 1.0 points	☐.☐
16. Mode of feeding a. Unable to eat without assistance = 0 points b. self-fed with some difficulty = 1 point c. self-fed without any problem = 2 points	☐

SELF ASSESSMENT

17. Do they view themselves as having nutritional problems? a. major malnutrition = 0 points b. does not know or moderate malnutrition = 1 point c. no nutritional problem = 2 points	☐
18. In comparison with other people of the same age, how do they consider their health status? a. not as good = 0.0 points b. does not know = 0.5 points c. as good = 1.0 points d. better = 2.0 points	☐.☐

ASSESSMENT TOTAL (max. 30 points): ☐☐.☐

MALNUTRITION INDICATOR SCORE		
≥ 24 points	well-nourished	☐
17 to 23.5 points	at risk of malnutrition	☐
< 17 points	malnourished	☐

Ref.: Guigoz Y, Vellas B and Garry PJ. 1994. Mini Nutritional Assessment: A practical assessment tool for grading the nutritional state of elderly patients. *Facts and Research in Gerontology.* Supplement #2: 15-59.