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Probiotic-directed modulation of gut microbiota is basal microbiome dependent

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ABSTRACT

As an effective means to improve quality of life and prevent diseases, the demand for probiotics and related products has increased in recent years. However, it is still unclear whether a particular probiotic strain will have similar beneficial effects on healthy adults from different regions. In this study, the probiotic *Lactobacillus casei* Zhang (LCZ) was consumed by healthy adults from six different Asian regions and the changes in gut microbiota were compared using PacBio single molecule, real-time (SMRT) sequencing technology based on samples collected before, during and after consumption of LCZ. Our results reveal that the effect of LCZ consumption on individuals was closely related to the composition of that individual's basal gut microbiota. A Gut Microbiota Variability Index (GMVI) was proposed to quantitatively compare the effects of LCZ on human gut microecology. Subjects from Xinjiang and Singapore regions had the highest and lowest GMVI, respectively. In general, consumption of LCZ increased the relative abundance of certain beneficial bacteria such as *Lactobacillus, Roseburia, Coprococcus* and *Eubacterium rectale*, while it inhibited growth of certain harmful bacteria such as *Blautia* and *Ralstonia pickettii*. In addition, consumption of LCZ was responsible for the conversion of some participants from *Prevotella copri/ Faecalibacterium prausnitzii* (PF) enterotype to *Faecalibacterium prausnitzii/Bacteroides dorei* (FB) enterotype and consistently increased the abundance of lactic acid bacteria in the gut. It also increased/enhanced phosphate metabolic modules, amino acid transport systems, and isoleucine biosynthesis, but conversely decreased lipopolysaccharide biosynthesis. These changes could have health benefits for healthy adults.

Introduction

For decades, the ability of probiotics to impart health benefits has prompted increasing scientific interest. The therapeutic effect of probiotic supplementation has been studied in a wide range of diseases, particularly in regard to gastrointestinal and metabolic disorders where the results have supported the potential use of probiotics as therapeutic agents.[1,2] Suggested mechanisms by which probiotics may benefit the gut environment and the health of the host include: improving intestinal barrier function through effects on the epithelium and mucus lining; producing antimicrobial substances; competing with pathogenic bacteria; and regulating luminal acidity.[3–5] Although researches have demonstrated positive effects of probiotic consumption on several health outcomes, the majority of published studies have focused on populations with specific health pathologies. Evidence supporting the health promoting effects of probiotics in healthy adults is limited and less consistent.[6,7] This may be as a result of the numerous confounding factors that exist. These include variation in consumer susceptibility to probiotic effects and significant differences in probiotic products (e.g. differences in microbial strains, the concentration of viable cells and product formulation).[8] To date, the effects on healthy adults from different regions has not been fully revealed for a single probiotic strain.

In recent years, the gut microbiota has received much attention as a potential risk factor for disease development, that has the potential to be manipulated. Results showed that some gut microbiota were

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associated with metabolic diseases such as obesity,[9] diabetes[10] and the occurrence and development of gastrointestinal diseases.[11–13] Other gut microbiota are involved in functional processes that are essential for homeostasis, such as digestion of otherwise indigestible nutrients, and production of vitamins and micronutrients.[14,15] In summary, the composition and changes in the gut microbiota are closely related to human health. Therefore, evaluation of the effects of probiotic consumption on gut microbiota could be used to estimate the efficacy of probiotics.

The development of high-throughput 16 S rRNA gene sequencing technology has accelerated our understanding of gut microbiota diversity. Illumina sequencing is the most widely used platform in research because it has the advantages of high sequencing throughput, low cost and high accuracy. However, the Illumina platform has a relatively short sequencing length. If it is applied for amplicon sequencing, the microbiota composition in the samples can only be detected at the genus level.[16] The Pacific Biosciences single molecule, real-time sequencing technology (SMRT) is a state-of-the-art tool that enables profiling of microbiota from environmental samples. The long reading length achievable using the PacBio SMRT sequencing platform provides an effective method for researchers to analyze the composition of bacteria in samples at the species level.[17,18]

Previous studies on the relationship between probiotics and human health have mostly focused on western societies, with only a few studies based on the Asian population. In this study, 106 healthy adults from six different regions in Asia were selected as research subjects, and the gut microbiota in healthy adults from different regions before and after of probiotic *Lactobacillus casei* Zhang (LCZ) consumption were evaluated based on PacBio SMRT sequencing technology. The probiotic strain (LCZ) we chose in the present study was originally isolated from naturally fermented mare's milk (also known as koumiss) collected in Inner Mongolia of China[19] and it possesses beneficial properties, including reducing hepatic inflammatory responses,[20] lowering of blood cholesterol,[21] producing anti-oxidation effect,[22] alleviating the respiratory and gastrointestinal abnormalities,[23] and improving the host immunity.[24] The aims of this study were to answer the following three questions: (1) What are the composition characteristics and differences in gut microbiota amongst healthy adults from different regions in Asia? (2) Does the same probiotic strain have similar beneficial effects on healthy adults from different regions? (3) Is the probiotic effect related to the individual basal gut microbiota composition of adults? The results of this study may guide the decision-making of consumers, researchers and manufacturers on choice of probiotic supplementation.

Results

Sequence coverage of bacterial community across all samples

We generated a dataset consisting of 2,395,377 raw sequence reads of full-length bacterial 16 S rRNA, with 5649 ± 5362 (in mean ± SD, range from 2917 to 14110) per sample. A total of 1,764,724 sequences were delimited through PyNAST alignment and 100% sequence identity clustering for further analysis. At a high threshold identity cutoff level of 97% sequence similarity, 413,475 OTUs were detected. After removing singleton OTUs, the average number of OTUs per sample was 1046 (range:371–2622; SD = 344). Each OTU was assigned to the lowest taxonomic level by homologous sequence alignment and clustering based on information extracted from the RDP, Greengenes and Sliva databases. Accordingly, 9.17% and 16.0% of the sequences were not assignable to genus and species levels. After the alpha diversity of each sample was quantified, the results showed that Shannon-Wiener diversity curves, but not rarefaction curves, reached the saturation phase (Figure S1), suggesting that the sequence depth obtained was adequate for all the samples, although additional new phylotypes would possibly be identified by further sequencing.

Global composition of gut bacterial communities amongst participants from six regions prior to consumption of the probiotic LCZ

The baseline gut microbiota composition of participants from different regions was determined from samples taken on day 0 (i.e before LCZ consumption). At the phylum level, a total of 15 bacterial phyla were identified in the day 0 samples. Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria and Tenericutes were

the five most dominant bacterial phyla (contributing 48.57%, 41.92%, 5.90%, 1.61% and 1.60% of the total number of sequences, respectively; Figure 1a). At the genus and species level, a total of 241 bacterial genera and 503 bacterial species were identified. *Prevotella* was the most abundant genus (contributing to 18.28% of the total number of sequences), and the abundances of *Bacteroides* (17.09%), *Faecalibacterium* (8.70%), *Eubacterium* (7.59%), *Blautia* (4.16%), *Ruminococcus* (3.24%), *Clostridium* (2.83%), *Succinivibrio* (2.43%), *Roseburia* (2.27%), *Alistipes* (1.49%), *Lactobacillus* (1.40%), *Collinsella* (1.34%), *Parabacteroides* (1.29%), *Phascolarctobacterium* (1.22%), *Dialister*(1.15%), *Megamonas* (1.08%) and *Oscillibacter* (1.06%) all exceeded 1% (Figure 1b). At the species level, 13 species with an average relative abundance of more than 1% were identified, including *Prevotella copri* (16.17%), *Faecalibacterium prausnitzii* (8.68%), *Bacteroides dorei* (5.31%), *Eubacterium rectale* (3.99%), *Blautia wexlerae* (2.57%), *Succinivibrio dextrinosolvens* (2.43%), *Bacteroides plebeius* (2.42%), *Bacteroides uniformis* (1.89%), *Prevotella stercorea* (1.47%), *Collinsella aerofaciens* (1.33%), *Phascolarctobacterium faecium* (1.14%), *Bacteroides coprocola* (1.12%) and *Oscillibacter valericigenes* (1.00%) (Figure 1c); the above species accounted for 49.53% of total number of reads.

The correlation between F/B index and BMI of subjects in different regions was not consistent (Figure 1d). Subjects from Indonesia, Inner Mongolia and Gansu showed a positive correlation, while those from Mongolia, Singapore and Xinjiang showed a negative correlation, although none of these correlations were statistically significant ($P > .05$). In order to study the core gut microbiota of adults from different regions, the OTUs that were shared by at least 90% of subjects in one region were defined as the 'core OTUs' of adults. We found that 51 OTUs met this threshold condition (Figure 1e). Amongst them, fourteen 'core OTUs' were found in participants from all six regions, of which eight OTUs had annotation results for *Faecalibacterium prausnitzii*. The remaining annotation results for the other six OTUs were *Parabacteroides distasonis*, *Eubacterium rectale*, *Collinsella aerofaciens*, *Dorea longicatena*, *Parabacteroides merdae* and *Bacteroides ovatus*, respectively. 'Core OTUs' with annotation results for *Ralstonia pickettii* and *Ruminococcus gnavus* only appeared in participants from Singapore. 'Core OTUs' with annotation results for *Blautia wexlerae* only appeared in participants from Singapore and

Indonesia, and 'core OTUs' with the annotation result for *Coprococcus comes* only appeared in participants from Mongolia and Gansu.

Correlations analysis between the composition of gut microbiota and dietary habits (Table S1) indicated that the intake frequency of some foods was closely related to the abundance of some bacteria in the gut microbiota (Figure S2). For example, the relative abundance of *Blautia wexlerae*, *Blautia obeum*, *Ruminococcus gnavus*, *Faecalibacterium prausnitzii*, *Eubacterium hallii* were significantly negatively correlated with the intake frequency of lamb, while they were significantly positively correlated with the intake frequency of seafood, rice, fruit, soybean, vegetables and fermented foods.

Differences in gut microbiota composition and enterotype characteristics of participants from six regions prior to LCZ consumption

The number of observed species (Figure 2a) and Shannon diversity index (Figure 2b) of the microbiota of participants from Singapore and Gansu were significantly lower than those from Indonesia, Inner Mongolia, Mongolia and Xinjiang ($P < .05$). There were significant differences amongst the six regions in the relative abundance of 156 species in the microbiota ($P < .05$); amongst these 55 species had an average relative abundance above 0.1% (Figure 2c, Table S2). Specifically, the gut microbiota of participants from Indonesia had a significantly higher abundance of *Faecalibacterium prausnitzii*, *Prevotella copri*, *P. stercorea* and *Escherichia/Shigella dysenteriae*, compared with participants from other regions. Similarly, the gut microbiota of participants from Inner Mongolia had a significantly higher abundance of *Bacteroides dorei*, *Eubacterium rectale* and *Bacteroides uniformis*; the gut microbiota of participants from Mongolia had a significantly higher abundance of *Eubacterium siraeum* and *Coprococcus comes*; the gut microbiota of participants from Singapore had a significantly higher abundance of *Fusicatenibacter saccharivorans*, *Megasphaera elsdenii*, *Bacteroides fragilis*, *Blautia faecis*, *Eubacterium dolichum*, *Bacteroides eggerthii* and *Klebsiella pneumoniae*; the gut microbiota of participants from Xinjiang had a significantly higher abundance of *Succinivibrio dextrinosolvens*, *Phascolarctobacterium faecium*, *Lactobacillus ruminis*, *Coprococcus eutactus* and *Vampirovibrio chlorellavorus*;
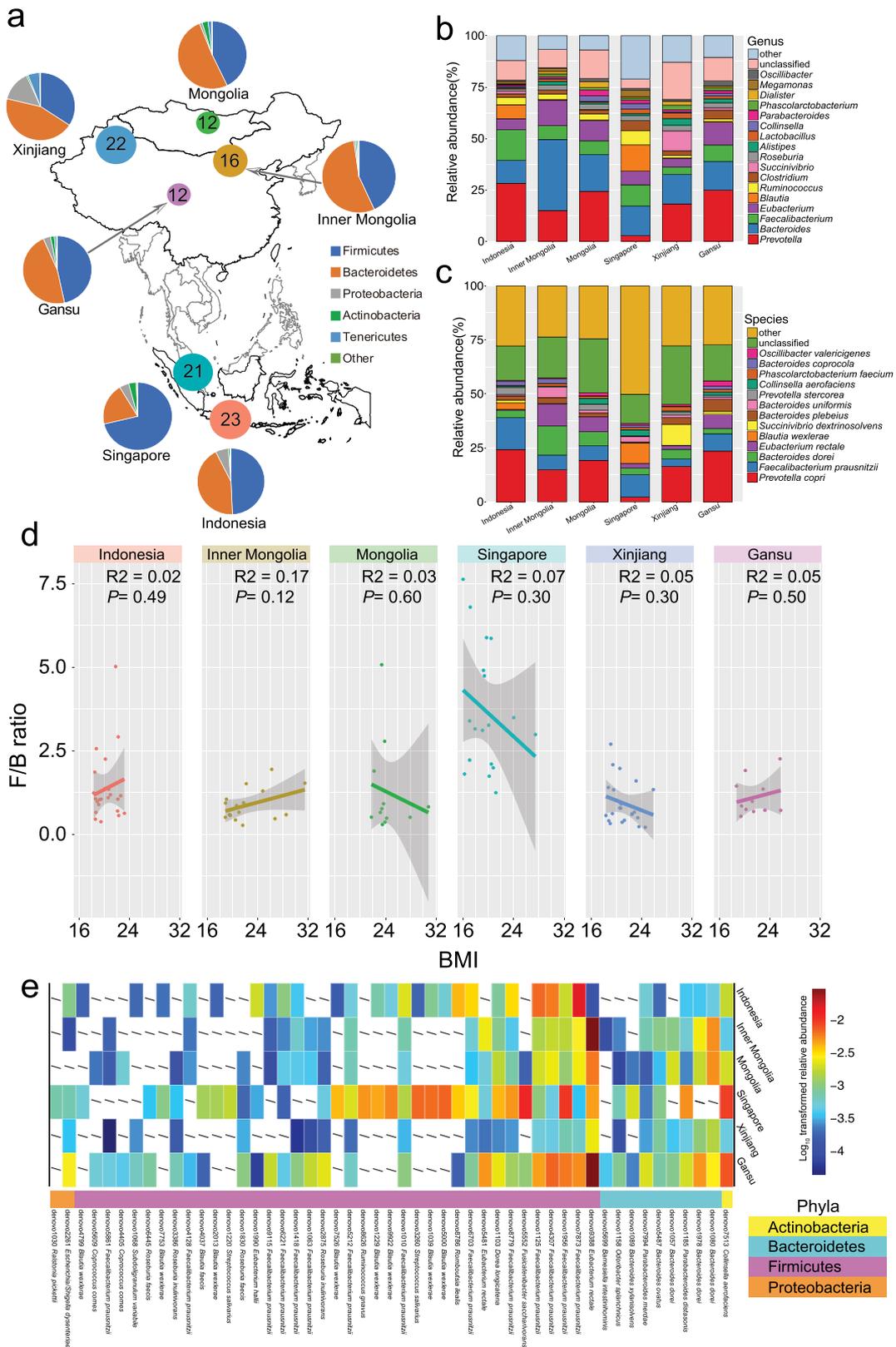
Figure 1. Basal composition characteristics of the gut microbiota of participants from six regions prior to consumption of the probiotic LCZ. a, b and c represent: phylum-level, genus-level and species-level bacterial composition, respectively, as determined using PacBio SMRT sequencing of 16 S rRNA genes. Each pie chart and column chart shows the mean composition of participants from each region. d: the relationship between F/B index and BMI index of participants from each region. e shows the core OTUs of gut microbiota in participants from each region. The color of squares represents the median of the relative abundance of each OTU.
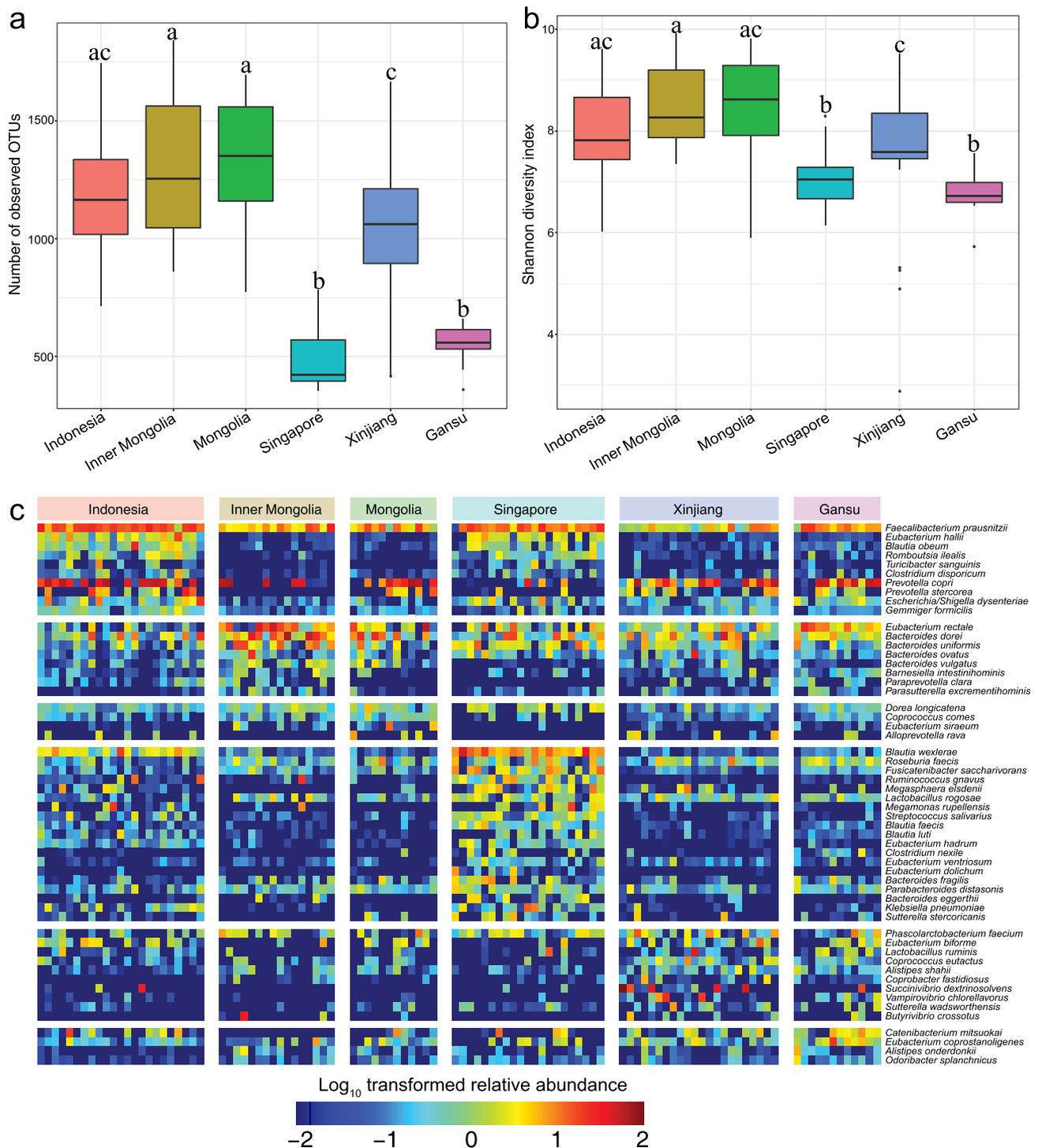
Figure 2. Comparison of α diversity indicators and species-level bacterial composition of the gut microbiota amongst participants from six regions prior to consumption of the probiotic LCZ. a and b represent: the number of observed OTUs and Shannon diversity index of the gut microbiota from participants from each region, respectively. Dots represent outliers from the respective groups. c: the gut microbiota that vary significantly in abundance amongst subjects from different regions. Species corresponding to the diagonal indicates that the average relative abundance of those species in this region are higher than those in the other five regions.

and the gut microbiota of participants from Gansu had a significantly higher abundance of *Catenibacterium mitsuokai*, *Eubacterium coprostanoligenes*, *Alistipes onderdonkii* and *Odoribacter splanchnicus*.

Significant clustering was observed and validated at species level. The two clusters could be divided into PC1-positive and PC1-negative regions ([Figure 3a](#)). Henceforth, the *Prevotella*

copri/*Faecalibacterium prausnitzii* rich microbiota of the PC1-positive group of participants will be referred to as PF-enterotypes (PF-type), whereas the *Faecalibacterium prausnitzii*/*Bacteroides dorei* rich microbiota of the PC1-negative group of participants will be referred to as FB-enterotypes (FB-type). The relative abundance of PF-type to FB-type participants in each region is visualized in Figure 3b. Notably, approximately 80% of participants from Singapore fell into the FB-enterotype group and most participants from Indonesia and Gansu fell into the PF-enterotype group. The abundances of the main microbial contributors to each enterotype revealed that *Prevotella copri* and *Bacteroides dorei* were strongly antagonistic to each other (Figure 3c, e). However, the relative abundance of *Faecalibacterium prausnitzii* was relatively high in both enterotypes (Figure 3d). The results of the dietary habits showed that there were differences in the intake frequency of some foods between the two enterotypes (Figure S3). For example, intake frequency of beef, mutton, seafood, eggs, fruits, wheat and rice was relatively high in the PF-enterotype group. In contrast, the intake frequency of pork, chicken and fermented foods was relatively high in the FB-enterotype group. However, these differences in the dietary intake frequencies of the two enterotypes was not statistically significant.

In order to verify the relationship between the main contributors of each enterotype and the overall composition of the gut microbiota, we mapped the abundance of *Prevotella copri*, *Faecalibacterium prausnitzii* and *Bacteroides dorei* to the principal coordinate analysis chart based on weighted UniFrac distances. Samples with a high relative abundance of *Prevotella copri* were mainly concentrated in the left upper corner of the principal coordinate analysis chart (Figure 3f), whereas the samples with high relative content of *Bacteroides dorei* were mainly concentrated in the bottom right corner of the principal coordinate analysis chart (Figure 3h). There was no significant correlation between the relative abundance of *Faecalibacterium prausnitzii* in the samples and the location of the spots (Figure 3g), indicating that the composition of gut microbiota of participants was largely determined by *Prevotella copri* and *Bacteroides dorei*.

Effect of consumption of the probiotic LCZ by participants from six regions on the community structure of their gut microbiota

In this study, the 424 samples were divided into 24 groups according to the six regions and four sampling times. Scatter plots (Figure 4a, b) with the average values of the first and second principal components based on unweighted UniFrac distance and weighted UniFrac distance were drawn, respectively. The results showed that samples from the same region had a significant clustering trend before and after consumption of LCZ. Changes in the gut microbiota of participants after consumption of LCZ were calculated based on weighted UniFrac distances. After consumption of LCZ, the community structure of the gut microbiota of participants from Gansu and Xinjiang tended to converge together; individual differences amongst participants in Inner Mongolia increased, whereas the community structure of gut microbiota from participants from Indonesia and Singapore showed little change (Figure 4c). Furthermore, the weighted UniFrac distances of samples at the four time points were analyzed statistically using a pairing calculation (Figure 4d). The statistical results showed that changes in gut microbiota of participants from Xinjiang and Mongolia were significantly greater than those for participants from Indonesia, Gansu and Singapore during LCZ intervention ($P < .05$); changes in gut microbiota of participants from Singapore were significantly less than for participants from the other five regions ($P < .05$). Changes of gut microbiota after consumption of LCZ were analyzed at the species level. The CH index calculated showed that samples at each time point could be divided into the two enterotypes. Following consumption of LCZ, the enterotype of some participants changed (Table 1). The total number of participants that were the FB enterotype increase throughout the time period when LCZ was being consumed. When consumption of LCZ stopped, the number of FB enterotypes decreased, although the final number was still higher than at day 0. Among these, the number of participants from Inner Mongolia that were FB enterotypes increased from 50% at the beginning of the experiment to 87.5% by the 14th day of LCZ consumption. Furthermore, all participants from Singapore that were the PF enterotype at the beginning of the experiment had become the FB enterotype by the 14th day of LCZ consumption. Fourteen days after LCZ withdrawal, one person from
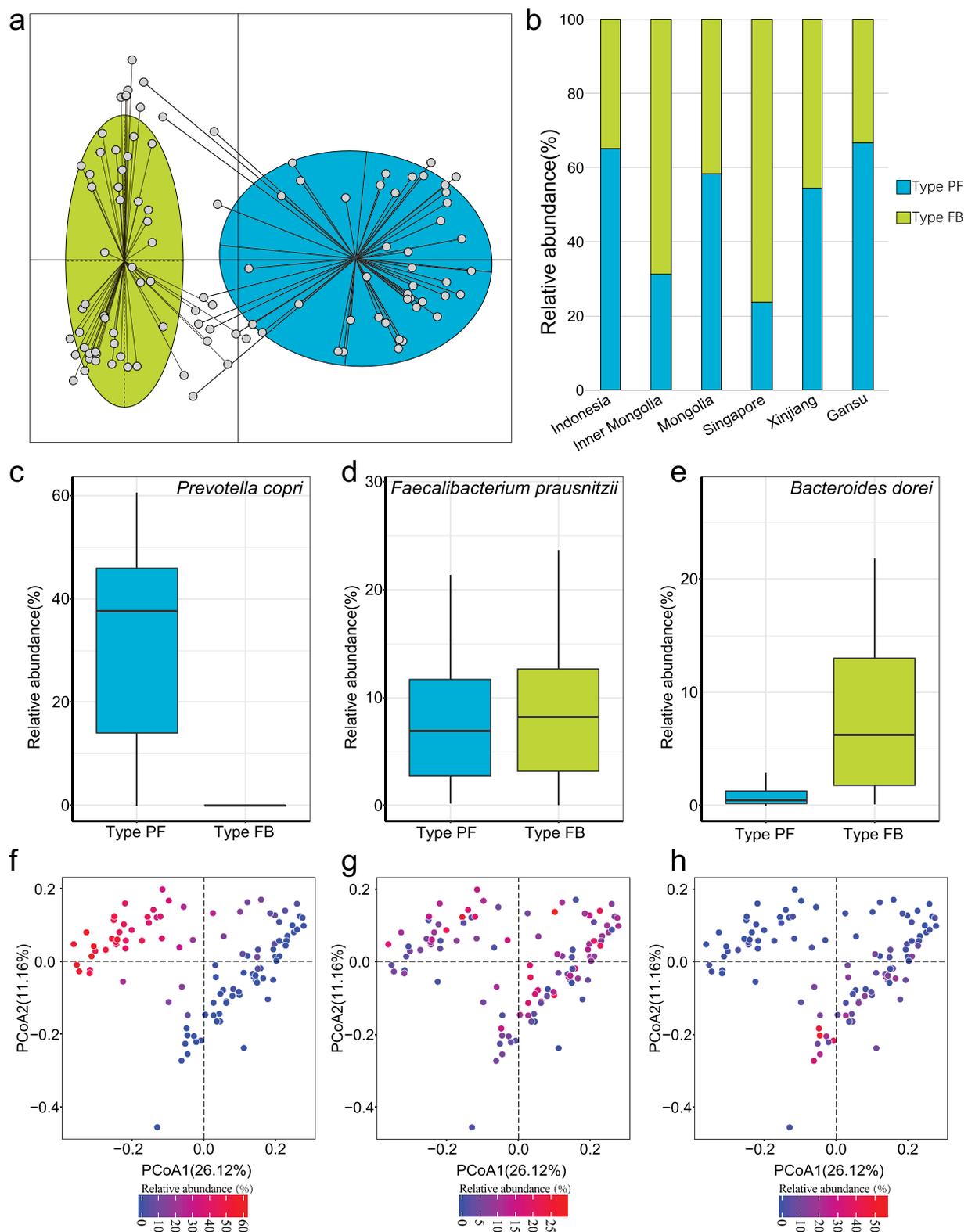
Figure 3. Enterotype analysis based on species-level bacterial composition of the gut microbiota of participants from six regions prior to consumption of the probiotic LCZ. a: all subjects divided into two different enterotypes. b: clustering of all the participants based on species composition data. The optimal number of clusters was chosen by maximizing the Calinski–Harabasz index and the clustering is displayed in the PCA plot. c, d and e represent: the relative abundances of *P. copri*, *F. prausnitzii* and *B. dorei* in each enterotype, respectively. f, g and h represent: the relationship between the relative abundances of *P. copri*, *F. prausnitzii*, *B. dorei* and the whole composition of gut microbiota, respectively.
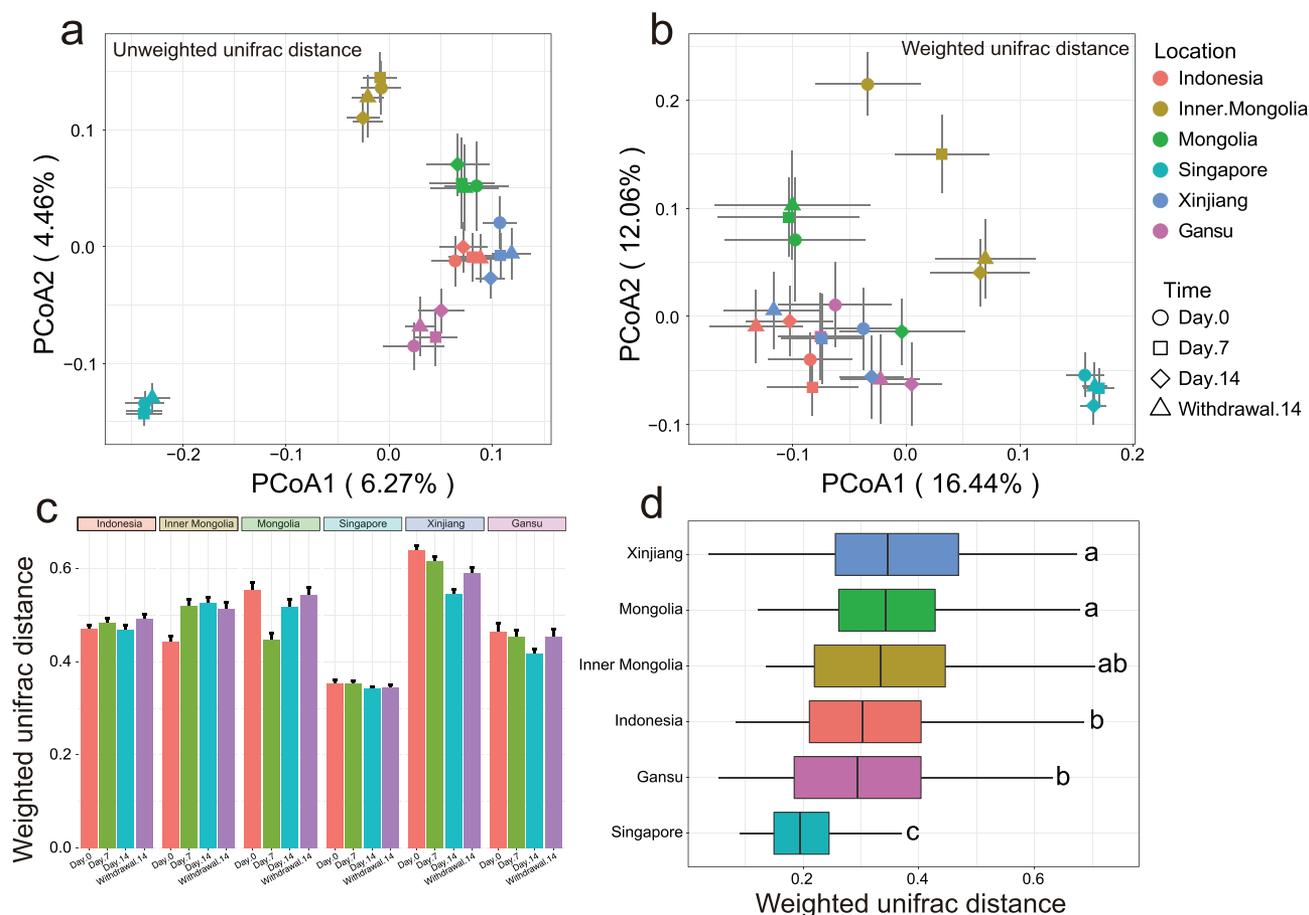
Figure 4. Effects of LCZ intake on the whole gut microbiota composition of participants from six regions. a and b represent: PCoA score plots based on unweighted and weighted UniFrac distance, respectively. Each point represents the mean principal component scores of all participants from one region at one time point, and the error bar represents the standard deviation. c: the height of each column represents the average distance amongst all samples at the same time point in a particular region. The error bar indicates the standard deviation of the distance between samples. d: the Weighed UniFrac distances of gut microbiota in participants before and after LCZ intake by paired calculation. There is no significant difference between groups with the same letter.

Table 1. Changes in the number of participants, from six regions, who were FB enterotypes at different times during an intervention with the probiotic LCZ.

Time	Number of FB enterotype of volunteers	Indonesia	Inner Mongolia	Mongolia	Singapore	Xinjiang	Gansu
Day 0	54	8	8	8	16	10	4
Day 7	58 ↑	8	12 ↑	6 ↓	18 ↑	10	4
Day 14	80 ↑	11 ↑	14 ↑	7 ↑	21 ↑	17 ↑	10 ↑
Withdrawal 14	62 ↓	8 ↓	13 ↓	6 ↓	20 ↓	9 ↓	6 ↓

Inner Mongolia and one person from Singapore had returned to the PF enterotype. The proportion of FB enterotype participants in Xinjiang and Gansu increased from 45.5% and 33.3% on day 0 to 77.3% and 83.3% on day 14, respectively, although the proportion of FB enterotype participants in these regions did not change significantly in the first 7 days of LCZ consumption. In contrast, only a few participants from Mongolia and Indonesia had enterotype changes during the experimental period.

Specific effects of consumption of the probiotic LCZ on gut microbiota of participants from different regions

We only analyzed gut microbiota with an average relative abundance of more than 0.1% at the species level following LCZ consumption and those that underwent continuous significant changes in abundance during the experiment are shown in Table 2. Amongst these, participants from

Table 2. Bacterial species in the gut microbiota of participants from six regions that underwent continuous and significant changes in abundance from the beginning to the end of the LCZ intervention.

Location	Species	Day 0	Day 7	Day 14	Withdrawal 14	P value
Indonesia	Blautia wexlerae	3.10 ± 0.84	3.04 ± 0.7	1.70 ± 0.54	1.52 ± 0.34	.029
	Eubacterium hallii	2.54 ± 0.69	1.79 ± 0.29	0.83 ± 0.16	0.98 ± 0.22	.001
	Eubacterium rectale	0.56 ± 0.24	1.38 ± 0.50	1.76 ± 0.38	1.94 ± 0.36	.002
	Blautia obeum	1.71 ± 0.83	1.08 ± 0.22	0.39 ± 0.12	0.31 ± 0.06	<.001
	Roseburia faecis	0.25 ± 0.08	0.55 ± 0.12	1.22 ± 0.48	1.10 ± 0.44	.003
	Eubacterium eligens	0.12 ± 0.04	0.33 ± 0.08	0.87 ± 0.28	0.29 ± 0.05	.007
	Sutterella stercoricanis	0.07 ± 0.03	0.18 ± 0.08	0.52 ± 0.20	0.43 ± 0.14	.018
	Lactobacillus rogosae	0.13 ± 0.05	0.19 ± 0.04	0.46 ± 0.07	0.39 ± 0.07	<.001
	Fusicatenibacter saccharivorans	0.16 ± 0.04	0.26 ± 0.09	0.45 ± 0.12	0.29 ± 0.05	.04
Xinjiang	Bacteroides ovatus	1.48 ± 1.23	0.62 ± 0.03	0.36 ± 0.08	0.12 ± 0.03	.003
	Subdoligranulum variabile	0.23 ± 0.06	0.25 ± 0.06	0.35 ± 0.07	0.21 ± 0.07	.031
	Bacteroides fragilis	0.10 ± 0.05	0.31 ± 0.15	0.60 ± 0.31	0.05 ± 0.04	.007
	Curvibacter lanceolatus	0.03 ± 0.02	0.23 ± 0.12	0.65 ± 0.52	0	.006
	Bacteroides xylanisolvens	0.30 ± 0.16	0.24 ± 0.02	0.14 ± 0.04	0.04 ± 0.01	.03
	Lactobacillus casei	0	0.01 ± 0.01	0.44 ± 0.27	0	<.001
Singapore	Clostridium leptum	0.23 ± 0.06	0.30 ± 0.07	0.34 ± 0.05	0.46 ± 0.09	.049
	Ralstonia pickettii	0.24 ± 0.16	0.18 ± 0.11	0.12 ± 0.03	0.21 ± 0.05	<.001
Mongolia	Lactobacillus rogosae	0.31 ± 0.12	1.74 ± 0.59	2.51 ± 1.51	0.15 ± 0.07	.016

Notes: the values in the table were percentages of relative abundance.

Indonesia and Xinjiang had the largest number of gut microbiota species that underwent continued significant changes in abundance. There was a continuous increase in the relative abundance of *Lactobacillus rogosae* in the gut microbiota of participants from Indonesia and Mongolia during the period of LCZ consumption. The relative abundance of a number of species underwent continuous and significant increases during the period of LCZ consumption and included. Specifically, *Roseburia faecis, Eubacterium eligens, Sutterella stercoricanis, Lactobacillus rogosae* and *Fusicatenibacter saccharivorans* increased continuously in participants from Indonesia; *Subdoligranulum variabile, Bacteroides fragilis, Curvibacter lanceolatus, Bacteroides xylanisolvens* and *Lactobacillus casei* increased continuously in participants from Xinjiang; *Clostridium leptum* increased continuously in participants from Singapore. However, other species underwent continuous significant declines in abundance during the period of LCZ consumption. Specifically, *Blautia wexlerae* continuously declined in participants from Indonesia; *Eubacterium hallii* and *Bacteroides ovatus* continuously declined in participants from Xinjiang; *Ralstonia pickettii* continuously declined in participants from Singapore. By 14 days after LCZ withdrawal, most species had returned to their relative abundances before LCZ intervention, except for *Blautia wexlerae, Eubacterium rectale, Blautia obeum, Bacteroides ovatus, Bacteroides xylanisolvens* and *Clostridium leptum.*

Relationship between changes in the gut microbiota of participants from six regions following consumption of the probiotic LCZ compared with the basal gut microbiota

The relative abundance of the gut microbiota of participants from some regions changed greatly during consumption of LCZ, however, in other regions they remained stable and changed very little. Specifically, during the period of LCZ consumption, ten species were found to significantly promote gut microbiota stability in participants. These included *Blautia wexlerae, Blautia obeum, Blautia faecis, Faecalibacterium prausnitzii, Eubacterium hallii, Ruminococcus gnavus, Turicibacter sanguinis, Klebsiella pneumoniae, Fusicatenibacter saccharivorans* and *Enterobacter cloacae* (Table 3). There were also two species that were not conducive to maintaining the stability of the gut microbiota, namely, *Bacteroides caccae* and *Alistipes shahii* (Table 3). The GMVI of subjects in Indonesia, Inner Mongolia, Mongolia, Singapore, Xinjiang and Gansu regions on day 0 were: 1.26 ± 0.18, −0.55 ± 0.21, −0.50 ± 0.19, −2.47 ± 0.31,0.03 ± 0.14 and −1.32 ± 0.21, respectively. Subjects from Xinjiang and Singapore regions had the highest and lowest GMVI, respectively.

Table 3. Species that significantly contributed to the impact of consumption of the probiotic LCZ on the gut microbiota of participants from six regions.

species	Day 7		Day 14	
	Correlation	P value	Correlation	P value
Blautia wexlerae	−0.34	<0.001	−0.42	<.001
Blautia obeum	−0.21	0.032	−0.4	<.001
Blautia faecis	−0.27	0.005	−0.38	<.001
Faecalibacterium prausnitzii	−0.21	0.031	−0.35	<.001
Eubacterium hallii	−0.24	0.015	−0.32	.001
Ruminococcus gnavus	−0.36	<0.001	−0.31	.001
Turicibacter sanguinis	−0.23	0.019	−0.3	.002
Klebsiella pneumoniae	−0.19	0.049	−0.25	.009
Fusicatenibacter saccharivorans	−0.23	0.019	−0.21	.031
Enterobacter cloacae	−0.2	0.041	−0.2	.039
Bacteroides caccae	0.25	0.011	0.24	.015
Alistipes shahii	0.25	0.009	0.26	.007

Effect of consumption of the probiotic LCZ on the metabolic function of the gut microbiota of participants from six regions

Using PICRUSt software and the KEGG database, 5668 KEGG homologues (KO, KEGG Orthologs) were predicted from all gut microbiota samples in this study. We firstly compared differences in gut microbiota function between the two enterotype populations, as the result, a total of 81 metabolic modules were found to vary significantly between the two enterotypes (Table S3). Amongst these, three modules (M00320, M00060 and M00080) were significantly more abundant in PF enterotypes and were related to lipopolysaccharide synthesis. Some modules involved in biosynthesis of essential amino acids were significantly more abundant in FB enterotypes, including modules associated with leucine biosynthesis (M00432), isoleucine biosynthesis (M00535) and histidine biosynthesis (M00026). In addition, Cobalamin biosynthesis (Vitamin B12; M00122), proline biosynthesis (M00015) and three presumed ABC transportation system (M00258, M00211, M00247) modules were also significantly more abundant in FB enterotypes.

During the period of LCZ consumption, seven metabolic modules underwent continuous and significant changes in abundance in FB enterotypes. Amongst these, the metabolic modules that continuously increased in abundance were: cytochrome o ubiquinol oxidase (M00417); fumarate reductase, prokaryotes (M00150); thiamine transport system (M00191); and

ArcB-ArcA (anoxic redox control) two-component regulatory system (M00456). In contrast, the modules that continuously decreased in abundance were: lipopolysaccharide biosynthesis KDO2-lipid A (M00060); the glutamate transport system (M00233); and the PTS system, N-acetylgalactosamine-specific II component (M00277).

There were 48 modules that underwent continuous and significant changes in abundance in PF enterotypes during the period of LCZ consumption (Table S4). Amongst these, 78.9% (30) of the modules that continuously increased in abundance belonged to the functional category of environmental information processing and included various phosphate and amino acid transport systems (M00229, M00237, M00230, M00225, M00236), two-component regulatory systems (M00506, M00449, M00445, M00471), and phosphotransferase systems (PTS) (M00283, M00275, M00273). There were also a further five modules that continuously increased in abundance which belonged to the categories of nucleotide and amino acid metabolism, specifically: homoprotocatechuate degradation (M00533); isoleucine biosynthesis (M00570); valine/isoleucine biosynthesis (M00019); ascorbate degradation (M00550); and GABA biosynthesis (M00136). Metabolic modules that continuously decreased in PF enterotypes included: CMP-KDO biosynthesis (M00063); lipopolysaccharide biosynthesis (M00060); the reductive acetyl-CoA pathway (M00377); the lipoprotein-releasing system (M00255); multiple sugar transport systems (M00216); aminoacyl-tRNA biosynthesis (M00360); pantothenate biosynthesis (M00119); and guanine ribonucleotide biosynthesis (M00050).

Characteristics of gut LAB in participants from six regions

The final sequencing quantity of LAB in each sample was 375 ± 20, as amplified by LAB specific primers. The Shannon diversity curve for each sample, based on the current sequencing quantity, tended to or reached a horizontal state (Figure S4), indicating that the current sequencing quantity was sufficient to reveal the composition of LAB in most samples, and met the needs of subsequent analysis.

At the genus level, five of the seven target LAB genera were detected in all samples, namely *Streptococcus*, *Lactobacillus*, *Enterococcus*, *Weissella*

and *Pediococcus*. The composition of LAB in samples from the six regions on day 0 is shown in Figure 5a. The predominant LAB in gut microbiota of all participants were *Streptococcus* and *Lactobacillus*. At the species level, 122 LAB were identified in total. The dominant LAB species included *L. ruminis* (26.68%), *S. salivarius* (22.3%), *S. thermophilus* (7.71%), *L. delbrueckii* (4.13%), *E. durans* (3.16%), *W. confusa*, (2.82%), *S. parasanguinis* (2.25%) and *L. casei* (2.18%) (Figure 5b). Alpha diversity analysis

showed that the abundance and diversity of LAB in the gut microbiota of participants from Inner Mongolia were the highest, and significantly higher than those from Indonesia and Singapore (Figure 5c, e). LefSe analysis found that there were significant differences in the relative abundance of 13 LAB species amongst the participants from the six regions. For example, *S. infantarius, W. confusa* and *E. hirae* were more abundant in the gut microbiota of participants from Indonesia; *S. parasanguinis, L. paralimentarius*
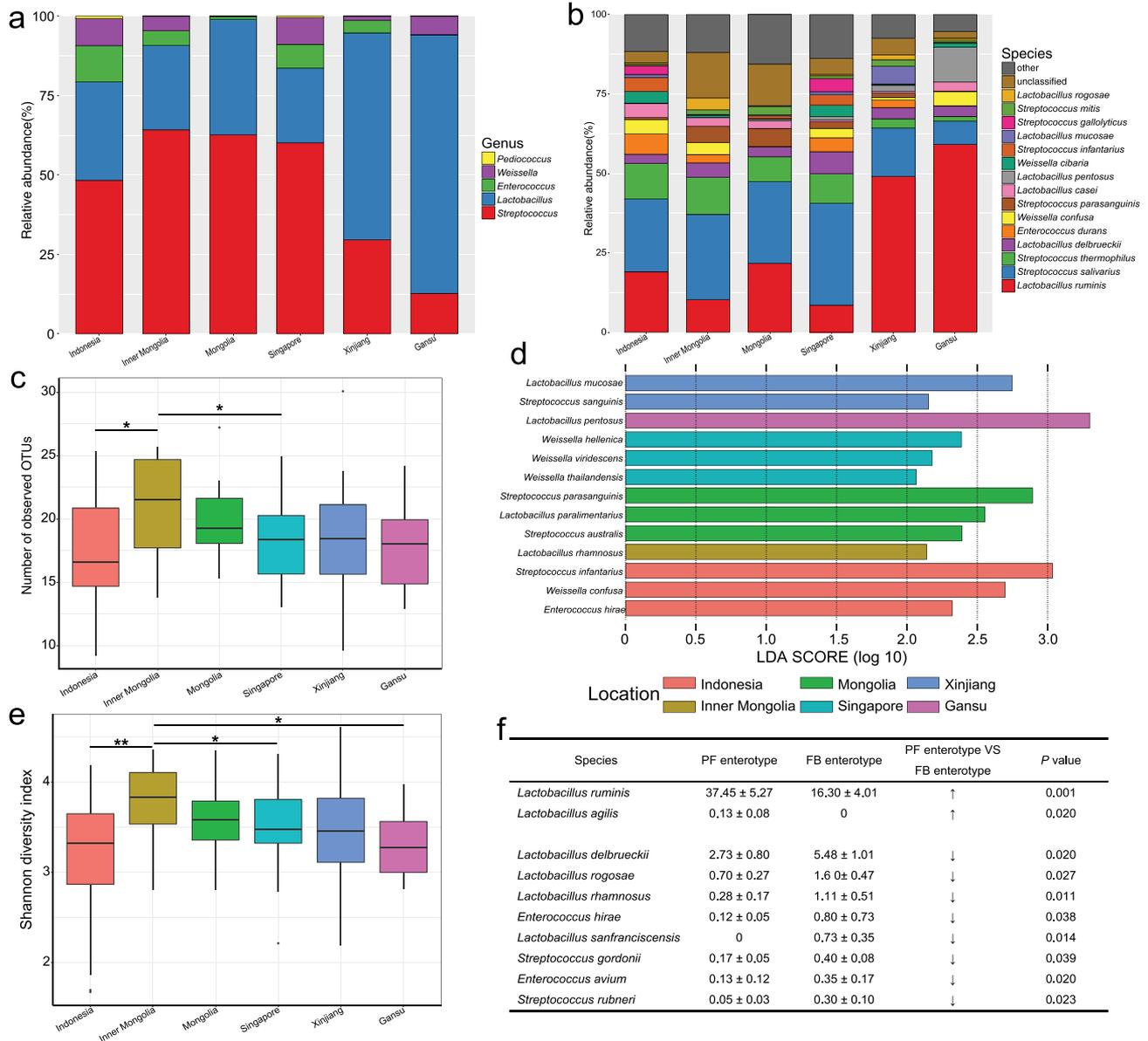

Figure 5. Composition characteristics of gut LAB in participants from six regions. a and b represent: genus-level and species-level gut LAB composition in participants from six regions, respectively. c and e represent: the number of observed OTUs and Shannon diversity index of gut LAB in participants from six regions, respectively. Dots represent outliers of the respective groups. d: LefSe analysis of gut LAB in participants from six regions at day 0. f: LAB species that with significantly relative abundance between PF and FB enterotypes.

and *S. australis* were more abundant in samples from Mongolia; *W. hellenica*, *W. viridescens* and *W. thailandensis* were more abundant in samples from Singapore. A total of ten LAB species, with an average relative abundance of more than 0.1%, varied significantly in abundance between the two enterotypes (Figure 5f). The LAB with a higher average relative abundance in PF enterotypes included *L. ruminis* and *L. agilis*, while the LAB with higher relative abundance in FB enterotypes included *L. delbrueckii*, *L. rogosae*, *L. rhamnosus*, *E. hirae*, *L. sanfranciscensis*, *S. gordonii*, *E. avium* and *S. rubneri*.

The effects of LCZ consumption on the α diversity of LAB in the gut of participants were evaluated at different time points during intervention. The observed species index demonstrated that the abundance of gut LAB in participants from all regions followed an upward trend over the period that LCZ was consumed (Figure 6a). In particular, the abundance of gut LAB in participants from Xinjiang increased significantly after 14 days of LCZ consumption ($P = .015$). When LCZ was withdrawal, the abundance of gut LAB in followed a downward trend in all participants except those from Inner Mongolia and Mongolia. Changes in LAB relative abundance in the gut microbiota of participants from different regions and with different enterotypes

after LCZ consumption were compared using paired Kruskal-Wallis tests at the species level (Table S5). Amongst participants from five regions (i.e. excluding the Gandu region), the relative abundance of *L. casei* changed significantly. It is worth noting that on the 7th day of LCZ consumption, the relative abundance of *L. casei* tended to reach its highest abundance in each group, and by the 14th day its relative abundance had always decreased to a certain extent. In order to reveal the reason for the decrease in the relative abundance of *L. casei*, we used ddPCR to quantify *L. casei* in all fecal samples (Figure 6b). This showed that the copy number of *L. casei* in fecal samples increased significantly during the period of LCZ consumption (from $10^{8.22\pm0.71}$/g on day 7 to $10^{8.45\pm0.83}$/g on day 14, $P < .05$); it also showed that after LCZ consumption, the decrease in relative abundance of *L. casei* was related to an increase in the abundance of other LAB.

Discussion

The composition of gut microbiota in healthy adults and the differences in their responses to intervention with the same probiotic are important in determining their roles in human health and wellbeing. We found that Firmicutes, Bacteroidetes, Proteobacteria,
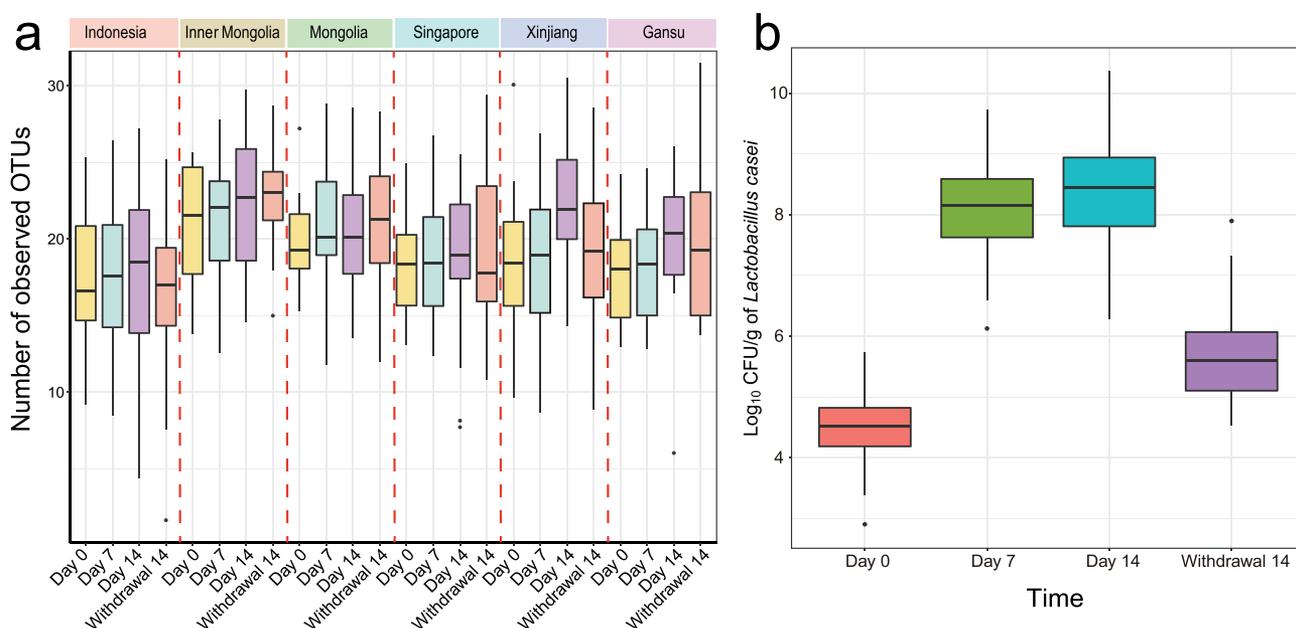

Figure 6. Effect of consumption of the probiotic LCZ on the gut LAB of participants from six regions. a: Changes in the number of observed OTUs of gut LAB in participants from each region at different times during the period of consumption of LCZ. b: The quantity of *Lactobacillus casei* as determined using ddPCR.

Actinobacteria and Tenericutes were the predominant phyla in the fecal microbiota of all participants, which was consistent with the results of many previous studies.[25,26] The F/B value is the most common indicator for the composition of gut microbiota. Turnbaugh et al., reported that obesity was related to an increase in the gut F/B value.[27] However, subsequent studies could not reproduce this finding.[28,29] In our study, correlations between the F/B value and BMI index was not consistent amongst participants from different regions; our data do not support a corresponding relationship between gut F/B value and obesity in healthy adults. At the species level, participants from different regions varied in their gut microbiota community structures, which were closely related to the dietary habits of people in the different regions; this is consistent with a previous study.[30] It is worth noting that *Faecalibacterium prausnitzii* was detected in the gut of all participants; this species is a specific anaerobic, gram-negative, non-spore-producing butyrate-producing bacterium.[31] As a potential probiotic for humans, *F. prausnitzii* has an immunomodulatory effect, and is helpful in alleviating the symptoms of diabetes.[32,33] The presence of *F. prausnitzii* in all healthy adults indicates that it may play an important role in maintaining the health of Asian adults.

Population stratification is a useful approach for better understanding complex biological problems in human health and wellbeing. The hypothesis that this stratification approach applies to the human gut microbiome, in the form of distinct community composition types termed enterotypes, was evaluated in this study. This indicated that variation in the gut microbiota of healthy Asian adults clustered into two enterotype groups that were driven by trade-offs between *P. copri/ F. prausnitzii* and *F. prausnitzii/B. dorei*. For the first time, we defined the bacteria that were representative of each enterotype at the species level. This is an advance on previous studies that were limited by the length of sequencing technology and could only identify gut representative bacteria at the genus level.[34–36] Although not statistically significant, we found some differences in dietary habits between the PF and FB enterotypes. For example, PF enterotypes consumed wheat, rice, eggs, fruits and seafood more frequently than FB enterotypes. Staple foods were their main source of carbohydrate. Previous studies have shown that the PF enterotypes were strongly dependent on carbohydrates in the diet.[37,38] In addition, eggs and chickens are known to contain high concentrations of vitamin A and vitamin B5, and that fruits and seafood are part of the Mediterranean diet, all of which favor the growth of *Prevotella* in the gut.[39,40] Our results are in accord with the notion that diet plays an important role in shaping an individual's enterotype.

Bacteroides and *Prevotella* are the main genera defining enterotypes and this has been reported in previous studies,[34,37] The results of the current strengthen this conclusion, i.e. that together *Prevotella copri* and *Bacteroides dorei* determine the overall composition of the gut microbiota of healthy Asian adults.

During the period of LCZ consumption, the gut microbiota of participants from some regions changed significantly at the species level. Amongst those increasing in relative abundance in some regions were *Eubacterium rectale, Roseburia faecis* and *Subdoligranulum variabile*, which are important short-chain fatty acid-producing bacteria;[41,42] in addition, the increase in relative abundance of *Clostridium leptum* is related to decreases in intestinal inflammation and severe alcoholic fatty liver disease.[43,44] Those species that declined included *Ralstonia pickettii* which is associated with obesity-related metabolic disorders. Previous research has shown that the abundance of *R. pickettii* increased in stool samples of obese subjects with pre-diabetes and type 2 diabetes and that fecal *R. pickettii* levels were correlated with plasma adiponectin levels, which are markers for impaired metabolic control.[45] Although not all the above-mentioned bacterial species underwent significant changes in abundance in all regions after intake of LCZ, it did have a positive impact on gut microbiota in participants from some regions. This was achieved via increasing the relative abundance of some beneficial gut microbiota and inhibiting the growth of some harmful bacteria. When LCZ was withdrawal, most of the gut microbiota that had undergone significant changes in abundance tended to return to levels prior to LCZ intervention. This implies that supplementation with probiotics may need to be an ongoing process in order to maintain positive gut microbiota changes in healthy adults.

Studies on the effects of probiotic supplementation on healthy adults have reached quite different conclusions;[6,46] it was thought that the variation in results may have been related to the type of probiotics and the dose.[47] Our results show that the basal gut microbiota is also an important factor that influences the effect of probiotics. Based on GMVI values calculated, we found that in regions where gut microbiota changed greatly after LCZ consumption, the basal GMVI of subjects in those regions was also higher. In particular, some short-chain fatty acid-producing gut bacteria, such as *F. prausnitzii*, *E. hallii* and *B. obeum*, encourage the gut microbiota to remain stable after LCZ consumption,[48–50] while *B. caccae*, an opportunistic pathogen, makes the gut microbiota more variable after LCZ indestion. The results show that individuals with healthier gut microbiota composition may have stronger resistance to new invasive bacteria. Therefore, the results of probiotic trials conducted in one population cannot be extrapolated to other populations that have substantially different basal gut microbiota.

Previous studies suggest that gut community composition and enterotype identity remains stable in healthy adults after short-term dietary changes.[37,51] However, consumption of the probiotic LCZ could cause a change in the gut enterotype of some consumers from the PF enterotype to the FB enterotype. As we required all participants to maintain their original dietary habits during the experiment, this suggests that there was a direct relationship between enterotype changes and probiotic consumption.

Functional analysis showed that there were significant differences between the two enterotypes in many metabolic modules. For example, three modules related to lipopolysaccharide metabolism were significantly more abundant in PF enterotypes than FB enterotypes, while many pathways related to human essential amino acid synthesis were significantly higher in FB enterotypes than PF enterotypes. Lipopolysaccharides are components of the cell membranes of gram-negative bacteria, and gut microbiota-derived lipopolysaccharides and systemic endotoxemia are involved in the onset and progression of atherosclerosis, inflammatory bowel disease, obesity and related metabolic diseases, and nonalcoholic steatohepatitis.[52–54] Essential amino acids are those amino acids that cannot be synthesized by the human body, or cannot be synthesized at a speed that meets requirements and must be provided from external sources. A lack of essential amino acids leads to a series of problems including metabolic disorders and a decline in immune resistance.[55] Considering that the biosynthesis pathway for lipopolysaccharide was enriched in PF enterotypes and various modules related to the synthesis of essential amino acids were enriched in FB enterotypes, we speculate that the composition of gut microbiota of FB enterotypes may be more beneficial to the health of adults. After LCZ consumption, the enterotype of many adults changed from PF to FB with a significant decrease in abundance of the lipopolysaccharide biosynthesis module in both enterotypes confirms the probiotic effect of LCZ.

LAB are an important component of beneficial microbiota in the human gut, and have attracted increasing attention due to their probiotic properties, contribution to promoting digestion and enhancing immunity, and ability to inhibit the growth of pathogenic bacteria.[56] Previous studies have reported that *L. ruminis* is the LAB species with the highest relative abundance in the gut of adults,[57] which is consistent with the results of this study. In addition, we also found that *S. salivarius*, *S. thermophilus*, *L. delbrueckii*, *E. durans* and *W. confusa* had high relative abundances in the gut of healthy Asian adults, which increased our understanding of the composition of LAB in the gut. Based on sequencing and the quantitative ddPCR results, we revealed that consumption of LCZ not only increases the abundance of *L. casei* in the adult gut, but also increases the abundance of other LAB, which may have health benefits.

Conclusions

There were substantial differences in gut microbiota amongst healthy Asian adults from different regions. The effect of LCZ consumption on individuals was closely related to the composition of that individual's basal gut microbiota. In summary, LCZ consumption had positive impacts on the gut microbiota via increasing the relative abundance of some beneficial gut microbiota, inhibiting the growth of some harmful bacteria, effectively increasing the abundance of beneficial LAB, increasing beneficial metabolic functions of the gut microbiota, and cause enterotype

changes in some individuals. Our study supports the hypothesis that consumption of probiotics by healthy adults, regulates the gut microbiota and produces probiotic effects.

Methods

Experimental strain

Packaged *Lactobacillus casei* Zhang (LCZ) powder was provided by Jinhua Yinhe Biotech (Jinhua, China) and the number of living bacteria in the probiotic LCZ preparation was 0.5×10^{10} CFU/g.

Participant recruitment

A total of 106 healthy adults from six different regions of Asia were recruited in this study. All participants were born and grew up in the sampling region, and were between 18 and 28 years old. Specifically, the subjects came from Inner Mongolia (16), Xinjiang (22), Gansu (12) in China and Mongolia (12), Singapore (21) and Indonesia (23), respectively (Figure1a). None of the subjects had gastric, gut, microbiota or metabolic diseases, and had not taken antibiotics in the 6 months prior to the experiment or during the experiment. In addition, in the first two weeks before LCZ consumption, all participants were asked to maintain their usual lifestyle and diet, but to refrain from consuming any other probiotic products or foods specifically labeled or marketed as probiotics throughout the study period. The participants answered a questionnaire that addressed their physiological characteristics, healthy condition and dietary intake in the two weeks before stool sampling began. The information of participants' age, Body Mass Index (BMI) and dietary habits in different regions are summarized in Table S1.

Consumption dose of probiotic LCZ and collection/preservation of fecal samples

During the experiment, all subjects took 2 g of LCZ powder packaged independently within 1 hour after lunch every day for 14 days. Human fecal samples were collected on day 0 (the day before the first day that subjects began consuming probiotic LCZ), day 7, day 14 and 14 days after

consumption of LCZ had stopped. On sampling days, the subjects collected about 10 g of fresh fecal material with a disposable sampling spoon, put them into a 50 ml sterile enzyme-free centrifuge tube, added about equal volume of DNA protection solution, and marked the samples. After the feces were fully mixed with the protective liquid, the sample tubes were sealed with sealing film, and temporarily stored in liquid nitrogen. Then, the samples were frozen at a collaborating laboratory in the sampling regions, and finally transported to the laboratory of the Inner Mongolia Agricultural University (IMAU) within a week to ensure consistency of subsequent experiments.

DNA extraction and target gene amplification

Genomic DNA was extracted from fecal samples at the laboratory of the IMAU using the QIAGEN DNA Stool Mini-Kit (QIAGEN, Hilden, Germany), following the manufacturers' instructions. The quality of the extracted genomic DNA was checked using agarose gel electrophoresis and spectrophotometric analysis (optical density at 260 nm/280 nm ratio). The final DNA concentration was above 100 ng/μL, while the 260 nm/280 nm ratio was between 1.8–2.0.

Bacterial full-length 16 S rRNA gene sequences were amplified from all genomic DNA samples by polymerase chain reaction (PCR) for SMRT barcode sequencing using forward 27 F (5′-AGAGTTTGATCMTGG-CTCAG-3′) and the reverse 1492 R (5′-ACCTTGTTACGACTT-3′) primers.[58] The lactic acid bacteria (LAB) primer was the L5 primer pair designed and screened by our laboratory.[59] This primer simultaneously amplifies seven common gut LAB (*Lactobacillus, Streptococcus, Weissella, Lactococcus, Pediococcus, Enterococcus* and *Leuconostoc*) using forward 15 F (5′-NNNGCTCAGGAYGAACGCYG G-3′) and reverse 687 R (5′-NNNCACCGCTACACA-TGRADTTC-3′) primers. A set of 16-base barcodes for every DNA sample was added to the forward and reverse PCR primers. The PCR amplifications were done as described previously.[60] The PCR programme was as follows: 95°C for 4 min; 30 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 30 s with a final extension at 72°C for 5 min. The amplification conditions for LAB were adjusted. The annealing temperature for LAB was 62°C and the number of amplification cycles was 26.

Quantitative analysis of *Lactobacillus casei* in samples was evaluated by Bio-rad Droplet Digital PCR (ddPCR).

Sequencing by SMRT

16 S rRNA gene amplicons were used to construct DNA libraries with the Pacific Biosciences SMRT bell™ template prep kit 1.0 as described previously.[58] Sequencing was done using P6-C4 chemistry on a PacBio RS II instrument in accordance with the manufacturer's instructions.

Bioinformatics analyses

The protocol RS_ReadsOfinsert.1, which was available in the SMRT Portal version 2.3, was applied to process the raw data. Restrictive filtering parameters were based on the following criteria: (i) minimum full passes of up to five; (ii) minimum predicted accuracy of 90. According to the length of the target fragment, the minimum and maximum read lengths of inserts were adjusted; the values of the above parameters for full-length 16 S rRNA were 1400bp and 1800bp, respectively. The minimum and maximum read lengths of LAB were 600bp and 1000bp, respectively. Before bioinformatic analysis, the primers and sequence barcodes were removed using in-house python scripts, and then all reads were sorted into different samples according to their barcodes.

Bioinformatics analysis was done on the extracted high-quality sequences using the Quantitative Insights Into Microbial Ecology (QIIME) package (version 1.7).[61] Briefly, the most abundant sequence from each cluster was selected as representative and aligned by PyNAST[62] and UCLUST[63] under 100% clustering of sequence identity. The unique sequence set was classified into operational taxonomic units (OTUs) under the threshold of 97% identity using UCLUST after the selection of the representative sequences. ChimeraSlayer[64] was applied to remove potential chimeric sequences in the representative set of OTUs. OTUs with only one sequence (singleton OTUs) were considered to originate from PCR errors and sequencing errors and were removed from all PacBio datasets. Each OTU was assigned to the lowest taxonomic level based on information extracted from the Ribosomal Database Project

(RDP, Release 11.5),[65] Greengenes (version 13.8)[66] and Sliva (Version 132)[67] to determine the phylogeny and relative abundance of the OTUs at a minimum bootstrap threshold of 80%.[68] The OTUtable was subsampled accordingly to adjust sampling depth of all samples using the multiple_rarefactions.py program in the QIIME pipeline. Alpha and beta diversity were calculated based on the *de novo* taxonomic tree constructed by the representative chimera-checked OTU set using FastTree.[69] Rarefaction estimators and Shannon-Wiener were calculated to evaluate sequence depth and microbial diversity, respectively.

Statistical analyses

Statistical analyses were done using the R package (http://www.r-project.org/) and Matlab (v2011b). Differences between groups in the diversity indices of gut microbiota were evaluated using the Mann-Whitney rank sum test or the Kruskal-Wallis test. False discovery rate (FDR) values were estimated using the Benjamini–Yekutieli method to control for multiple testing.[70] *P*-values less than 0.05 were considered statistically significant. To assess the community structure of microbiota from different samples, principal coordinate analysis (PCoA) based on the weighted and unweighted UniFrac distances derived from the phylogenetic tree was done.[71] The correlation between Firmicutes/Bacteroidetes (F/B) index and BMI index of subjects in different regions was established by linear regression analysis. Enterotype analysis was done based on the method described by Arumugam et al.,[34] the optimal number of enterotypes was determined using the Calinski-Harabasz (CH) index. Spearman's rank correlation was used to evaluate the co-occurrence relationships amongst gut microbiota and dietary habits. In order to reveal the influence of LCZ consumption on the gut microbiota, changes in the weighted UniFrac distances of gut microbiota from the same participant at different times during the LCZ consumption period were correlated, at the species level, with the basal gut microbiota (day 0). In order to quantify variability in the gut microbiota of participants throughout LCZ consumption, we defined

and used a 'gut microbiota variability index' (GMVI) for participants under LCZ intervention using the species identified as being either conducive or unfavorable for maintaining gut microbiota stability. The GMVI was calculated using the formula:

$$I = \frac{\sum_{i \in N} A_i}{N} - \frac{\sum_{j \in M} A_j}{M}$$

where I is the calculated GMVI value of gut microbiota at day 0 before intervention. A_i and A_j are the relative abundance for each of the species that were found from earlier analysis to be either unfavorable (A_i) or conducive (A_j) to maintaining the stability of gut microbiota under LCZ intervention, respectively. N and M were the numbers of unfavorable (A_i) and conducive (A_j) species, respectively. According to this formula, the larger the I value is, the easier it is to change the community structure of the gut microbiota following LCZ consumption. Using this formula, we calculated and compared the GMVI of each participant from the six regions.The functional profiles of the gut microbiota were predicted using PICRUSt[72] and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Based on the methods of Feng et al.,[73] we used a Z value ≥1.6 (90% confidence according to normal distribution) as the detection threshold to determine whether there were significant differences in metabolic pathways or metabolic modules. Graphic presentations were generated using the R 'ggplot2' package.[74]

Abbreviations

LCZ	*Lactobacillus casei* Zhang
SMRT	Single Molecule, Real-Time
GMVI	Gut Microbiota Variability Index
OTU	operational taxonomic unit
LAB	lactic acid bacteria
PCR	polymerase chain reaction
KEGG	Kyoto Encyclopedia of Genes and Genomes
BMI	Body Mass Index

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Authors' Contributions

HZ and ZS designed the experiments. FZ, WL, RL, WK and JH performed the experiments. QH analyzed the data. QH and YL wrote the main manuscript. All authors read and approved the final manuscript.

Availability of Data and Materials

Raw sequence data were made publicly available online through MG-RAST project number mgp91010 (http://meta genomics.anl.gov/linkin.cgi?project= mgp91010).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Ethics Approval

Written informed consent was obtained from all subjects. The study protocol was approved by the Ethical Committee of the Inner Mongolia Agricultural University (Hohhot, China).

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