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Full Paper

Gut microbial stability in older Japanese populations: insights from the Mykinso cohort

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Gut microbiota imbalance plays an important role in the pathogenesis of various diseases. Here, we determined microbe–microbe interactions and gut microbiome stability in a Japanese population with varying body mass indices (BMIs) and enterotypes. Using 16S ribosomal RNA gene sequencing, we analyzed gut microbial data from fecal samples obtained from 3,365 older Japanese individuals. The individuals were divided into lean, normal, and obese groups based on their BMIs. They were further categorized according to their gut microbiota enterotypes: *Bacteroides* (enterotype B), *Prevotella* (enterotype P), and *Ruminococcus* (enterotype R). We obtained data on different host factors, such as age, BMI, and disease status, using a survey questionnaire evaluated by the Mykinso gut microbiome testing service. Subsequently, we evaluated the co-occurrence network. Individual differences in BMI were associated with differences in co-occurrence networks. By exploring the network topology based on BMI status, we observed that the network density was lower in the lean group than that in the normal group. Furthermore, a simulation-based stability analysis revealed a lower resistance index in the lean group than those in the other two groups. Our results provide insights into various microbe–microbe interactions and gut microbial stability and could aid in developing appropriate therapeutic strategies targeting gut microbiota modulation to manage frailty.

Key words: gut microbiota, body mass index, microbe–microbe interactions, gut microbial stability

INTRODUCTION

The prevalence of obesity has nearly tripled worldwide since 1975. According to the World Health Organization (WHO), over 650 million adults were obese in 2016 [1]. Furthermore, an annual study on national health and nutrition in Japan by the Ministry of Health, Labour and Welfare reported that male obesity has been increasing since 2013. According to the 2019 statistics, 33.0% of males are overweight, with an average body mass index (BMI) of 25 or more, which is 4.4 points higher than that in 2013 [2]. The study using data from past Japan National Health and Nutrition Survey demonstrated a rapid increase in the prevalence of obesity among older Japanese men from 1973 to 2016 [3]. However, in 2016, the percentages of older men and women who had a BMI of 20 kg/m^2 or less and tended to be undernourished were 13.4% and 22.4%, respectively [4]. A previous study also speculated about the sudden increase that has been observed in the prevalence of underweight among women in the 65–69 and 70–79 years age groups in Japan [5]. In addition, according to a previous cardiovascular health survey, approximately 12% of the Japanese population is considered frail [6], a condition that appears to increase with age, notably after the age of 75 [7].

Obesity and overweight are the major risk factors for noncommunicable diseases, increasing the likelihood of disability and death [8]. The medical costs associated with obesity-related disorders have risen dramatically and are expected to rise further. Moreover, being underweight is an independent predictor of increased morbidity and mortality because it is thought to result from body wasting [9, 10]. Individuals with a lower normal-BMI range (18.5–19.9) have a statistically higher risk of mortality among older Japanese individuals [9]. Moreover, some reports suggest that weight gain is linked to a good prognosis for diseases such as heart failure, and this phenomenon is known as the obesity paradox. Given the link between old-age frailty

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and sarcopenia, a high degree of weight loss may be detrimental to health [11]. The extent to which "obesity" and "thinness" are manageable, the health impacts, and age-dependent changes in their effects have not yet been fully established when considering the prognosis for life [11]. Japan is now among the world leaders in terms of longevity, with an increasing average life expectancy accompanied by a sharp increase in the ≥65 year-old population to 28% [12]. Because this population is susceptible to high health and economic burdens, timely and effective measures for managing their well-being are required.

Several studies have found strong associations between gut microbiota and the energy–balance equation [13, 14]. Clinical and basic research have demonstrated that the gut microbiota plays a critical role in the pathogenesis of obesity development [13–16]. Furthermore, evidence indicates a strong association between gut microbiota and being underweight [14, 17]. Because the gut microbiota is linked to the progression of obesity and underweight, which are becoming increasingly common in Japan's aging society, influencing the gut microbiota is gaining attention as a novel therapeutic approach. However, because the gut microbiota varies between individuals and can be classified into different enterotypes [18], it is debatable whether a uniform intervention would benefit individuals with different enterotypes. We previously reported a strong association between gut microbiota and being underweight [17]. As older adults in Japan are commonly underweight and have attracted attention, finding clues to preventing underweight via the modulation of gut microbiota is important.

The gut microbiota appears to exert a myriad of positive functions in metabolism, host protection, and the gut-brain axis [19]. Statistically significant differences in gut bacterial community diversity, composition, phenotype, function, and ecological networks have been reported based on 16S rRNA gene sequencing data. The resulting profiles were linked to BMI; however, they were sex specific [20]. The gut microbiome is often noted for its ecological stability, which is critical for host health and well-being because it ensures that beneficial symbionts and their associated functions are maintained over time [21, 22]. Therefore, it is speculated that investigating how microbial interactions influence microbial community dynamics can help comprehend the ecological stability of the microbiome. Ecological modeling of microbiomes that considers such interactions is a crucial step towards a better understanding of community function [23], anticipating dynamics [22], and rationally developing interventions that change community structure and function [24]. Previous studies have reported the association of microflora instability with poor health and frailty [25], particularly in older individuals [26]. In light of these findings, analyzing the interplay of microbes in the gut and simulating temporal changes in gut microbiota have considerable relevance. Therefore, we examined the data obtained from the Mykinso cohort, one of the largest cohorts of gut microbiota data in Japan and part of the product services of Cykinso, Inc. (Tokyo, Japan).

We hypothesized that microflora stability could be used to assess the frailty of older individuals. To test this hypothesis, in this study, we aimed to determine microbe–microbe interactions and gut microbiome stability in an older Japanese population with varying BMIs and enterotypes. Our findings could be significant because Japan has the highest proportion of older adults globally and older Japanese adults over 75 years of age are extremely frail [7].

MATERIALS AND METHODS

Study population

The study initially included 4,414 individuals who were >64 years old and registered in the Mykinso cohort from January 2017 to October 2021. Among the registered individuals, 856 were excluded from the study due to the presence of duplicate samples and samples from time points other than the first time point in longitudinal studies. Furthermore, 114 individuals were excluded due to unknown BMI, and 83 individuals were excluded due to lack of compliance with the survey questionnaire. Ultimately, 3,361 individuals were included in the study (Fig. 1). All participants provided written informed consent for enrollment, and the study was conducted according to the principles of the Declaration of Helsinki. The study was approved by the Cykinso Research Ethics Committee (no. LD-001-04 and LD-002-03) and registered with the UMIN Clinical Trials Registry (no. UMIN000028887 and UMIN000028888).

BMI stratification

The participants were categorized according to the WHO BMI classification as lean (BMI <18.5), normal (18.5≤ BMI <25), or obese (25≤ BMI) [21].

Common disease status

Using an original survey (Supplementary Table 1), information on the disease statuses of the participants was collected by the Mykinso gut microbiome testing service. The original survey included questions on lifestyle, bowel habits, and diseases. Individuals were scored "positive" for a disease if they replied yes to any original survey question, "negative" if they replied no, and "unknown" if data were unavailable across all original surveys.

Fecal sampling, DNA extraction, sequencing, and sequencing data analysis

We performed fecal sampling, DNA extraction, sequencing, and sequencing data analysis according to the protocol described by Kameoka *et al.* [27]. Fecal samples were collected using brush-type collection kits containing guanidine thiocyanate solution (TechnoSuruga Laboratory, Shizuoka, Japan), transported at ambient temperature, and stored at 4°C. DNA was extracted from the fecal samples using a DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The amplicons of the V1V2 region were prepared using a forward primer (16S_27Fmod: TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG AGR GTT TGA TYM TGG CTC AG) and reverse primer (16S_338R: GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GTG CTG CCT CCC GTA GGA GT). Sequencing libraries were prepared according to the 16S library preparation protocol provided by Illumina (San Diego, CA, USA). Libraries were sequenced in a 250 bp paired-end run (500 cycles) using MiSeq Reagent Kit v2 (Illumina).

Bioinformatics analysis

We performed data processing and taxa assignment based on the QIIME 2 pipeline (version 2020.8) [28] according to the following steps: (1) joining paired-end reads, filtering, and denoising using the DADA2 algorithm and (2) assigning taxonomic information to each amplicon sequence variant using

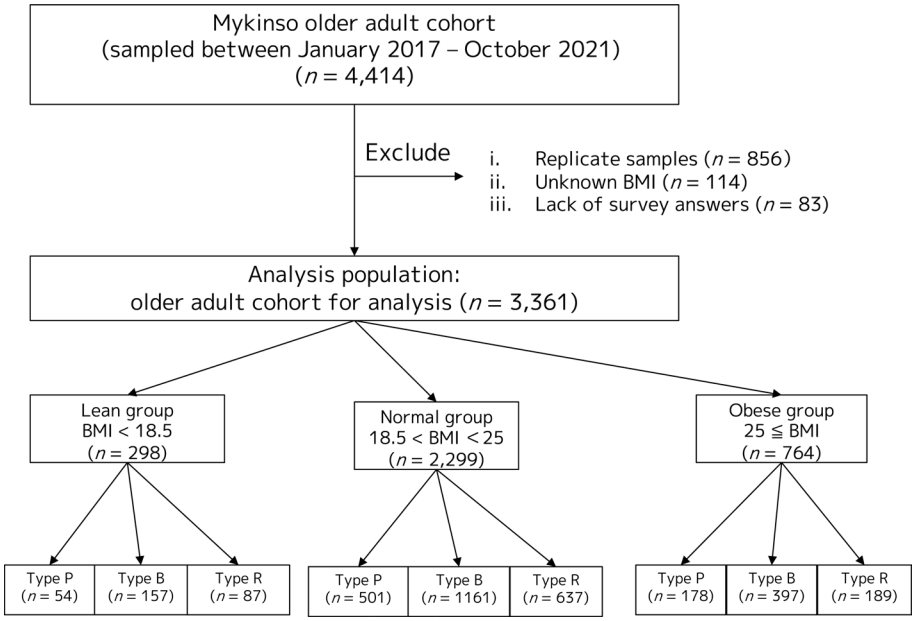

Fig. 1. Study population. Types P, B, and R represent enterotypes P (*Prevotella* enriched), B (*Bacteroides* enriched), and R (*Ruminococcus* enriched), respectively. BMI: body mass index; n: number of individuals.

a naive Bayes classifier in the QIIME 2 classifier. The classifier was trained using a robust taxonomy simplifier for SILVA (arts-SILVA), originally developed from the 16S rRNA taxonomy dataset based on SILVA 138 [17]. Relative abundance (RA) was calculated as the number of sequenced reads for each taxon in a sample and standardized by the total number of sequences generated for each sample. Only non-rare taxa that were present in at least 30% of the cohort and had an RA of at least 0.01% in at least one sample were included in the analyses. Sequence counts for taxa that did not meet these requirements were aggregated into an "Other" category. These filtering requirements were applied at the genus level.

Statistical analysis

Enterotyping

Samples were clustered using the Bray–Curtis distance and partitioning around medoid clustering [17]. The optimal number of clusters was estimated using the Calinski–Harabasz (CH) index. Finally, enterotypes were assigned based on the RA values of cluster centroids, encoded as enterotypes B (*Bacteroides* enriched), P (*Prevotella* enriched), and R (*Ruminococcus* enriched).

Evaluation of genera cross-correlation using SparCC

We used the SparCC network inference approach [29] to infer the co-existence networks of bacterial communities from each group. We then utilized the relative abundance at the genus level to compute associations and prepared one network for each group. We selected nodes and edges based on the determined cutoff point for the correlation level with a p-value <0.01 (permutation test with 200 permutations).

To further evaluate the topological features of each network, we used the NetShift software to identify driver nodes between case-control association networks [30]. We focused on identifying key attributes (e.g., nodes, clusters, and edges). We then detected driver nodes from comparisons between different BMI stages (normal vs. obese and normal vs. lean) among enterotypes B, P, and R.

Simulation-based stability analysis

Microbial community population temporal dynamics were simulated using a generalized Lotka–Volterra model [31]. The model was evaluated using numerical integration with the "lsoda" function in the "miaSim" R package [32]. Growth rates were assigned to each species from a uniform distribution from >0 to 1 so all species could show positive growth. Carrying capacities were assigned to each species by drawing from either a β distribution, which allows the simulation of a range of distributions from a uniform distribution (with the coefficients α=1 and β=1) to an increasingly uneven distribution (with the coefficients α=1 and β >1), or from a log-normal distribution to simulate an uneven distribution. Carrying capacity distributions were scaled to range between 1 and 100. The interaction matrix, which determines the topology of the metacommunity network, was assigned from correlation coefficients estimated by the SparCC program.

The measure of deviation from the baseline for each time point was determined based on two stability properties: resistance and resilience. Resistance refers to the ability of a system to remain mostly unchanged following a disturbance. This property is quantified by the maximal deviation from the reference state caused by a disturbance [33]. Resilience is the ability of a system to return to the reference state after a disturbance. For its quantification, we used the definition proposed in a previous study [34]. Resilience is computed as an index over time, with values ranging between −1 and 1.

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To investigate the associations between stability properties and host factors (age, BMI, disease status), we used multiple linear regression to explain each stability property (resistance and resilience) from the host disease status. The significance of each parameter was estimated by comparing the models with and without the corresponding factor in predicting each stability property using the "glm" function in the R "stats" package. The p-values were corrected using the Benjamini–Hochberg procedure.

RESULTS

Study population

A total of 4,414 older Japanese participants were enrolled in the study. After excluding participants lacking BMI data, 3,361 participants were included in the analysis and divided according to their BMI into lean, normal, and obese groups (Fig. 1). Baseline characteristics of the participants, including age, sex, BMI, and disease status, are shown in Table 1. Mean age significantly differed among the three groups, albeit by small margins (lean 72.5 ± 6.8, normal 72.2 ± 5.9, obese 71.3 ± 5.8; $p<0.001$). Mean BMI significantly differed among the groups (lean 17.16 ± 1.27, normal 21.97 ± 1.71, obese 27.3 ± 2.26; $p<0.001$). In addition, the percentage of females was significantly higher in the lean group than in the normal and obese groups (lean 77%, normal 54%, obese 47%; $p<0.001$; Table 1). The percentage of metabolic diseases, including cardiovascular disease, diabetes, dyslipidemia, and hypertension, was significantly higher in the obese group than in the lean and normal groups ($p<0.001$ for both; Table 1).

Enterotype

The 3,361 samples were clustered using genus abundance profiles. The CH index showed a clear global maximum at three clusters in each group dataset (Supplementary Fig. 1). In all group datasets, *Bacteroides* (enterotype B), *Prevotella* (enterotype P), and *Ruminococcus* (enterotype R) were the drivers of the three enterotypes (Supplementary Fig. 2). In the normal group dataset, 1,161, 501, and 637 participants possessed enterotypes B, P, and R, respectively. The numbers of participants possessing enterotypes B, P, and R in the lean group dataset were 157, 54, and 87, respectively, and those in the obesity group dataset were 397, 178, and 189, respectively. In all three groups, enterotype B was the most enriched (Fig. 1 and Table 1).

SparCC

Microbial interaction networks in the guts of the normal, obese, and lean participants were constructed using a SparCC algorithm. We then used NetShift to identify the driver nodes in each case-control association (lean vs. normal and obese vs. normal among enterotypes B, P, and R; Fig. 2). In each comparison, we built and compared the network association for each state and obtained some topological parameters, such as the network density, cluster coefficient, and average path length (Table 2). Together, they are called global graph properties because they provide insights into the overall organization of the network and enable the assessment of its modularity [30].

Table 2 shows that we detected a reduced density of the network in the lean condition in lean-normal comparisons for every enterotype (almost less than half the normal value). Thus, the low network density indicated microbial communities composed of scarcely connected groups. Moreover, the clustering coefficient quantifies the tendency of a graph to be divided into subunits. In other words, a microbial network with a higher number of independent units of associated microbes is expected to have a higher clustering coefficient value [30]. Our study showed consistent, remarkable differences in this parameter between comparisons of the lean and normal groups for every enterotype (almost less than half the normal value; Table 2).

Figure 2 shows our results for driver nodes and edge connections from the pairwise comparisons analyzed in this study. In enterotype B, edge connections of driver nodes comprising *Bacteroides*, *Collinsella*, and *Erysipelotrichaceae* were exhibited only in lean participants. In enterotype P, edge connections of driver nodes comprising *Prevotella*, *Collinsella*, and *Streptococcus* were only exhibited in lean participants. In enterotype R, edge connections of driver nodes comprising *Ruminococcus* and *Bifidobacterium* were exhibited only in lean participants.

Table 1. Characteristics of the study population

	Lean group (n=298)	Normal group (n=2,299)	Obese group (n=764)	p-value
Age (years)	72.5 (6.8)	72.2 (5.9)	71.3 (5.8)	<0.001
Females	228 (77%)	1,234 (54%)	362 (47%)	<0.001
Body mass index (kg/m^2)	17.16 (1.27)	21.97 (1.71)	27.30 (2.26)	<0.001
Enterotype B	157 (53%)	1,161 (51%)	397 (52%)	0.2
Enterotype P	54 (18%)	501 (22%)	178 (23%)	
Enterotype R	87 (29%)	637 (28%)	189 (25%)	
Common diseases	243 (82%)	1,903 (83%)	687 (90%)	<0.001
Gastrointestinal disease	106 (36%)	606 (26%)	205 (27%)	0.003
Liver disease	8 (2.7%)	72 (3.1%)	39 (5.1%)	0.027
Cardiovascular disease	22 (7.4%)	236 (10%)	112 (15%)	<0.001
Diabetes	17 (5.7%)	227 (9.9%)	120 (16%)	<0.001
Dyslipidemia	46 (15%)	561 (24%)	261 (34%)	<0.001
Hypertension	48 (16%)	814 (35%)	433 (57%)	<0.001

n: number of individuals; data show mean ± standard deviation (SD) values.

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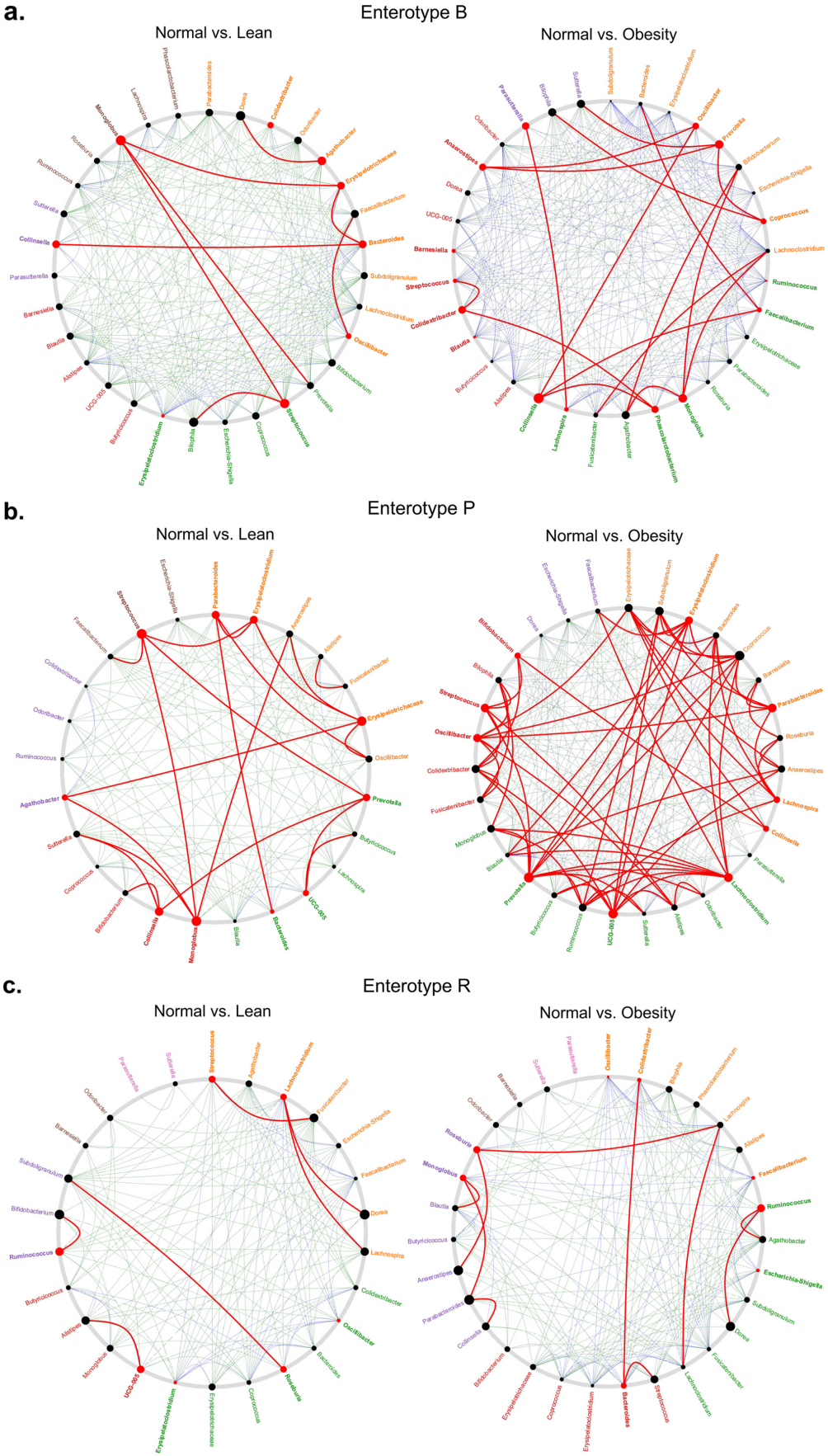

Fig. 2. Microbial interaction networks and driver nodes from pairwise comparisons of the groups based on BMI (normal vs. lean and normal vs. obesity) among enterotypes B (a), P (b), and R (c). Driver nodes were obtained using NetShift analysis; those shown in red indicate that the edges are exclusive to case data. All comparisons are based on a control-case order (normal vs. lean or normal vs. obesity). BMI: body mass index.

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Table 2. Network statistics for all pairwise comparisons between groups (normal, obesity, and lean)

		Host factors			
Enterotype	Case–Control comparisons	BMI status	Density	ClusterC	AvgPath
B	Normal vs. Lean	Normal	0.50	0.61	1.50
		Lean	0.12	0.31	2.86
	Normal vs. Obesity	Normal	0.49	0.60	1.51
		Obesity	0.30	0.44	1.80
P	Normal vs. Lean	Normal	0.37	0.39	1.67
		Lean	0.11	0.12	3.19
	Normal vs. Obesity	Normal	0.38	0.41	1.64
		Obesity	0.23	0.44	2.04
R	Normal vs. Lean	Normal	0.41	0.61	1.72
		Lean	0.13	0.44	2.75
	Normal vs. Obesity	Normal	0.38	0.58	1.72
		Obesity	0.15	0.47	2.31

The left column indicates network statistics. AvgPath: Average Path; ClusterC: Cluster coefficient, and Density; BMI: body mass index.

Simulation-based stability analysis

We simulated each enterotype microbial temporal variation using generalized Lotka–Volterra (gLV) dynamics (Supplementary Fig. 3a–3c). Non-rare genera subsampled from a metacommunity were used to produce local communities, and community population dynamics were simulated until steady-state abundances were reached. We then evaluated the stability properties of the ecosystems using mathematical criteria from dynamical systems theory.

Multiple linear regression analyses revealed that for enterotype P, the lean group had a significantly lower resistance value (coefficient=−0.12 [−0.18 to −0.05], p<0.001) than the normal group (Table 3 and Fig. 3). Additionally, for enterotype B, the lean group had a significantly lower resilience value (coefficient=−0.02 [−0.03 to −0.00], p=0.042) than the normal group (Table 3). We did not observed significant associations between stability indices and BMI status for enterotype R (Table 3).

DISCUSSION

Life expectancy has increased worldwide, leading to an increased incidence of frailty. Several studies have demonstrated the potential role of gut microbiota on frailty pathophysiology. In this large observational study, we studied the interplay of BMI, age, and disease status in an older adult Japanese cohort. We demonstrated that gut microbiota enterotypes and BMI are the key determinants for microbe–microbe interactions and microbiota stability in the older Japanese population. Our findings could be informative in the development of therapeutic approaches aimed at reducing frailty and involving the modulation of gut microbiota.

In this study, the proportion of females was considerably higher in the lean group than those in the normal and obese groups. This trend toward lean women (BMI <25 kg/m^2) has previously been reported in Japan [5]; however, its precise cause is not known. Previous studies on the distribution of gut bacterial communities among healthy individuals reported a higher abundance of genus *Bifidobacterium* in the gut microbiome of Japanese individuals than that in the gut microbiome of other individuals [17, 35–37]. Similarly, we found that enterotype B was the most enriched in the Japanese cohort analyzed in the present study.

Microorganisms form ecosystems through interspecies interactions (such as reciprocity and competition). In addition, it is known that gut microbiota ecosystems affect our health; therefore, understanding the gut microbial network is important in the field of medicine. Predicting microbial associations from microbial abundance data is known as network inference, and this is widely applied in bioinformatics and is beginning to be adopted in ecology [25, 38]. In addition, co-occurrence networks have also been used to build dynamic models of microbial communities. A dynamic model consists of the gLV equation and makes it possible to simulate the temporal changes in the abundance of community members. Application of the Lotka–Volterra model to metagenomic analysis has been shown to be successful for predicting the temporal dynamics of microbiota in the presence of known interspecific interactions (quantified microbial interactions) [30]. Therefore, we performed a realistic simulation using the gLV equation to evaluate the co-occurrence network of intestinal flora and its stability [39].

Improving microbiota resilience could have significant health implications [40]. Moreover, a genus-level correlation network has different microbe–microbe interactions associated with age and BMI [17]. SparCC have been estimated from microbiome data collected using a case-control design for interactions between bacterial species [29]. A previous study [39] reported that when the interaction between bacterial species is estimated using relative-amount data, the coefficients obtained by SparCC and the gLV model have high similarity. The co-occurrence coefficients between core genera and other minor genera of the microbial community network are frequently used to evaluate the keystone bacterial species and the interactions among the community members [39]. In the present study, we calculated the SparCC interaction coefficient for each cohort stratified by enterotype and BMI and compared the network association for each BMI status using a NetShift analysis. In a microbial community, density corresponds to the proportion of observed microbial associations (edges) out of all theoretically possible associations (all the nodes in the network). Therefore, a greater density value indicates higher crosstalk among the resident microbes represented in the network nodes [31]. This behavior was expected due to the scarce

Table 3. Comparison of the stability values across the BMI groups at each enterotype

Host factors	Host parameters	Enterotype	Resilience Coefficient (multivariable)		Resistance Coefficient (multivariable)	
BMI	Normal	B	——		——	
	Obesity		0.000 [−0.01 to 0.01], p=0.872		0.000 [−0.03 to 0.04], p=0.792	
	Lean		−0.020 [−0.03 to −0.00], p=0.042	*	0.040 [−0.01 to 0.10], p=0.111	
Age	60S		——		——	
	70S		0.010 [−0.00 to 0.02], p=0.199		0.010 [−0.03 to 0.04], p=0.742	
	80S or more		0.010 [−0.01 to 0.02], p=0.547		−0.030 [−0.08 to 0.03], p=0.319	
Sex	Female		——		——	
	Male		0.000 [−0.01 to 0.01], p=0.548		0.010 [−0.02 to 0.04], p=0.438	
Gastrointestinal disease	Absence		——		——	
	Presence		0.000 [−0.01 to 0.01], p=0.644		0.010 [−0.02 to 0.04], p=0.46	
Metabolic disease	Absence		——		——	
	Presence		0.000 [−0.01 to 0.01], p=0.756		−0.020 [−0.05 to 0.01], p=0.199	
BMI	Normal	P	——		——	
	Obesity		0.010 [−0.01 to 0.02],p=0.381		−0.040 [−0.08 to 0.00], p=0.067	
	Lean		0.010 [−0.01 to 0.04], p=0.348		−0.120 [−0.18 to −0.05], p<0.001	*
Age	60S		——		——	
	70S		−0.000 [−0.02 to 0.01], p=0.827		0.010 [−0.02 to 0.05], p=0.521	
	80S or more		−0.010 [−0.04 to 0.01], p=0.213		0.020 [−0.03 to 0.08], p=0.41	
Sex	Female		——		——	
	Male		0.000 [−0.02 to 0.01], p=0.804		0.010 [−0.03 to 0.04], p=0.711	
Gastrointestinal disease	Absence		——		——	
	Presence		0.000 [−0.01 to 0.02], p=0.57		−0.020 [−0.05 to 0.02], p=0.374	
Metabolic disease	Absence		——		——	
	Presence		0.000 [−0.01 to 0.01], p=0.911		0.030 [−0.01 to 0.06], p=0.148	
BMI	Normal	R	——		——	
	Obesity		−0.010 [−0.02 to 0.01], p=0.42		−0.000 [−0.04 to 0.03], p=0.807	
	Lean		0.020 [−0.01 to 0.04], p=0.184		−0.040 [−0.08 to 0.01], p=0.131	
Age	60S		——		——	
	70S		0.000 [−0.01 to 0.02], p=0.85		0.010 [−0.02 to 0.04], p=0.705	
	80S or more		−0.010 [−0.03 to 0.01], p=0.225		0.030 [−0.01 to 0.07], p=0.152	
Sex	Female		——		——	
	Male		0.010 [−0.01 to 0.02], p=0.419		−0.010 [−0.04 to 0.01], p=0.345	
Gastrointestinal disease	Absence		——		——	
	Presence		0.000 [−0.01 to 0.02], p=0.599		0.010 [−0.02 to 0.04], p=0.417	
Metabolic disease	Absence		——		——	
	Presence		0.000 [−0.01 to 0.02], p=0.744		−0.030 [−0.05 to 0.00], p=0.058	

To account for variations in participant characteristics or disease factors between the BMI groups, we estimated the statistical significance based on multiple linear regression corrected for age, gender, and disease status using a coefficient test followed by Benjamini–Hochberg correction. *p<0.05. BMI: body mass index.

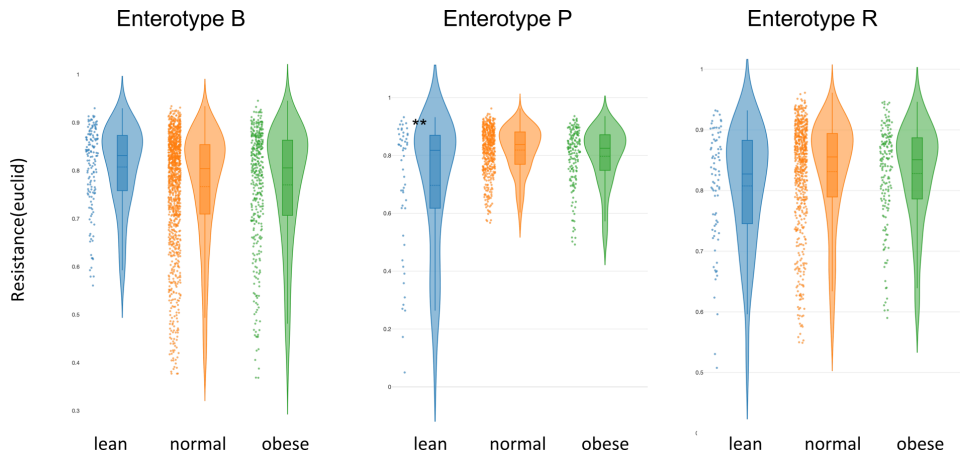

Fig. 3. Effect of overweight and underweight on simulation-based resistance value. Resistance values range from 0 (low) to 1 (high). **p<0.001 by multiple regression coefficient tests followed by Benjamini–Hochberg correction.

resilience of the system, as a poorly connected network is less robust to changes than high-density networks [30]. This behavior is in agreement with our results for the density and clustering coefficient in the lean status.

In many diseases, a set of key microbial groups likely acts as a "driver" for facilitating several changes in the microbial community structure and hence becomes an essential factor for understanding the microbial basis of the disease [30]. In our NetShift analysis, we detected driver taxa for facilitating BMI differences present in our older Japanese cohort. This driver genus captures several ecological insights that will be explained next. The edge connections of driver nodes comprising *Bacteroides*, *Collinsella*, and *Erysipelotrichaceae* among enterotype B have been related to metabolic interactions involving L-lactate and monosaccharides [41]. The edge connections of driver nodes comprising *Prevotella*, *Collinsella*, and *Streptococcus* among enterotype P have been related to metabolic interactions involving NH_3 and monosaccharides [41]. The edge connections of driver nodes comprising *Ruminococcus* and *Bifidobacterium* among enterotype R have been related to metabolic interactions involving Omega-6 fatty acid and Vitamin Bs (Vitamin B2, B6, B7, B12) [41]. In agreement with our findings, *Prevotella* was also decreased in older adults with frailty in an older Chinese Cohort [42]. In addition, other studies showed that the association with frailty was strongest for sugars added during food production [43]. These results indicate that decreased *Prevotella* occupation and abnormal monosaccharide metabolism could be closely related to frailty progression.

Furthermore, we performed a simulation-based stability analysis and calculated the gut microbial stability for each enterotype-body type among the older individuals using the interaction coefficient of each cohort obtained by SparCC estimation and the individual gut microbial composition according to the gLV model. The simulation-based stability analysis revealed a lower resistance index, which is one of the gut microbial stability indices [33], in the lean group than those in the other two groups, even after controlling for the covariation in age, sex, or disease status between the BMI groups, especially for enterotype P. Furthermore, being underweight is thought to result from body wasting [9, 10]. However, whether the low resistance or the instability of the gut microflora is a result of therapeutic interventions to treat frailty or caused by being underweight has yet to be explored. Further research is needed to understand the gut microbiome ecosystem and develop a novel method of microbiome manipulation to prevent frailty.

Many diseases, including inflammatory bowel disease, liver cirrhosis, rheumatoid arthritis, type 2 diabetes, and cardiovascular disease, are influenced by gut microbiota imbalance. However, to date, the mechanism by which gut microbiota instability affects disease progression remains unclear. Our findings shed light on gut microbial instability as a new marker for disease progression, in addition to gut microbial differences. Furthermore, our findings provide insights into various microbe–microbe interactions, as well as gut microbial stability in older Japanese populations. These findings could assist in determining gut microbial modulations for designing novel therapeutic approaches for obesity and frailty.

This study has some limitations that should be considered when interpreting the findings. First, this case-control study was limited to an evaluation of bacterial flora stability by correlation-based methods, hindering estimation of the directionality of ecological interactions. Therefore, a longitudinal study to estimate microbial interactions with more accuracy is necessary. Second, data from the Mykinso cohort may or may not be representative of the Japanese population. However, this study uses one of Japan's largest microbiome databases, and the use of this database eliminates bias caused by the small sample size. Third, we did not exclude from our dataset individuals who had common diseases because it is rather normal for some or most individuals to have one or more diseases, such as back pain and hypertension, when the target population is older adults (>64 years old). Therefore, the confounding effects of the prevalence of certain diseases on the association of microbiome stability and BMI classifications cannot be ruled out.

In conclusion, we demonstrated the microbe–microbe interactions and gut microbiome stability in older Japanese people and suggested an association between the reductions in microbiota resilience and BMI. Furthermore, we showed that changes in BMI are frequently associated with changes in a core set of covarying taxa. Our findings also revealed a low resistance index in the lean group. These findings could be useful for determining the types of gut microbiota and specific gut microbial target species for successful gut microbiota modulation in the development of appropriate therapeutic strategies to manage frailty and promote healthy aging.

DATA AVAILABILITY

All data analyzed and used in this study are available from the corresponding author upon reasonable request.

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