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ORIGINAL ARTICLE

A pilot study to examine the association between human gut microbiota and the host's central obesity

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Key words
continental population groups, diet, metabolic syndrome, microbiota, obesity.

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Abstract

Background and Aim: Perturbance in the composition of human gut microbiota has been associated with metabolic disorders such as obesity, diabetes mellitus, and insulin resistance. The objectives of this study are to examine the effects of ethnicity, central obesity, and recorded dietary components on potentially influencing the human gut microbiome. We hypothesize that these factors have an influence on the composition of the gut microbiome.

Methods: Subjects of Chinese (n = 14), Malay (n = 10), and Indian (n = 11) ancestry, with a median age of 39 years (range: 22–70 years old), provided stool samples for gut microbiome profiling using 16S rRNA sequencing and completed a dietary questionnaire. The serum samples were assayed for a panel of biomarkers (interleukin-6, tumor necrosis factor alpha, adiponectin, cleaved cytokeratin 18, lipopolysaccharide-binding protein, and limulus amebocyte lysate). Central obesity was defined by waist circumference cut-off values for Asians.

Results: There were no significant differences in Shannon alpha diversity for ethnicity and central obesity and no associations between levels of inflammatory cytokines and obesity. The relative abundances of *Anaerofilum* (P = 0.02), *Gemellaceae* (P = 0.02), *Streptococcaceae* (P = 0.03), and *Rikenellaceae* (P = 0.04) were significantly lower in the obese group. From principle coordinate analysis, the effects of the intake of fiber and fat/saturated fat were in contrast with each other, with clustering of obese individuals leaning toward fiber.

Conclusion: The study demonstrated that there were differences in the gut microbiome in obese individuals. Certain bacterial taxa were present in lower abundance in the group with central obesity. Fiber and fat/saturated fat diets were not the key determinants of central obesity.

Introduction

Obesity and obesity-related diseases are becoming important public health issues worldwide.[1] In Singapore, 4 in 10 adults aged 18–69 years were overweight according to the 2010 Singapore's National Health Survey. Of the 10 adults, 1 would be considered obese (body mass index [BMI] ≥ 30 kg/m^2). This rising trend of obese population has almost doubled since 1992.[2] Obesity is associated with insulin resistance (IR) and non-alcoholic fatty liver disease (NAFLD).[3] In recent years, the impact of NAFLD is becoming more significant globally,[3] with non-alcoholic steatohepatitis (NASH) being an increasingly common indication for liver transplantation[4] and liver cirrhosis.[5]

A growing body of evidence links obesity and IR with "dysbiosis" of gut microbiota.[6,7] This may be related to the improved ability of obesity-associated gut microbes to harvest energy from diet.[8] Clinical studies in obese populations have also shown that weight loss programs and diet restrictions alter underlying microbiota.[9] There are, however, conflicting results on the pattern of dysbiosis or the microbiota diversity in obesity.[9,10]

Although there are much data to support the ethnic phenotypic differences in IR,[11] the association between microbiota population and apparent phenotypic differences in people with a metabolic syndrome remains largely unexplored. With the majority of microbiota studies conducted using Caucasian populations, there is little information in the literature on the gut microbiota profile of Asian populations. Sordillo and colleagues found that

white race/ethnicity was associated with lower diversity but higher *Bacteroidetes* coabundance scores.[12] We found that *Faecalibacterium prausnitzii*, *Sutterella stercoricanis*, and *Bacteroides denticanum* were characteristic of Chinese, Malays, and Indians, respectively.[13]

Dietary intake is a well-recognized factor that has been associated with alterations in the gut microbiome. Röytiö *et al.* reported that consumption of fiber and fat amounting to the recommended dietary intake was associated with greater species richness and lower abundance of *Bacteroidaceae* in overweight pregnant women.[14] In addition, higher microbiota richness was linked to higher microbiota stability on increased dietary fiber intake, as well as higher proportions of *Prevotella* and *Coprococcus* species.[15] In yet another study, gut microbiome biomarkers were found to be correlated with clinical response to low fermentable oligosaccharides, disaccharides, monosaccharides, and polyols diet efficacy in childhood irritable bowel syndrome.[16]

While the changes in the gut microbiota seem important in the regulation of host metabolism, it has been shown that increased levels of endotoxemia (secondary to intestinal bacterial products translocating the gut wall) can influence insulin sensitivity.[17] Interestingly, endotoxemia-inducing lipopolysaccharide (LPS) infusion in normal-weight mice predisposed these mice to similar weight gain as those fed a high-fat diet only.[18] This mechanism of bacterial translocation has also been implicated in the pathogenesis of NASH.[19] Adipokines have also been known to play a role in modulating systemic inflammatory responses that contribute to obesity-linked cardiovascular diseases.[20]

To the best of our knowledge, there is currently no Southeast Asian study examining the gut microbiota profile in association with obesity. This is the first study to interrogate the intricate interplay between ethnicity, host metabolic disturbance, and dietary influence on the human gut microbiome. This study will also examine host inflammatory response and endotoxemia to central obesity. A better understanding of the disease pathogenesis and contributing factors may lead to improved therapeutic strategies in applying microbiome interventions.

Methods

Study participants. This is a single-center, prospective, cross-sectional pilot study of subjects with or without central obesity. The study population comprised healthy volunteers and type 2 diabetes mellitus (DM) patients. Central obesity was defined as waist circumference ≥ 90 cm in men and ≥80 cm in women for Asians, as defined by World Health Organization (WHO). Healthy volunteers were defined as having no family history or past history of autoimmune disease, obesity, DM, gastrointestinal cancers, inflammatory bowel disease, irritable bowel syndrome, anxiety, and depression. Subjects with no DM were confirmed by the absence of impaired glucose tolerance on fasting blood glucose. The BMI of all subjects was ≥18 kg/m^2.

The following subjects were excluded: those with viral hepatitis or of immune-compromised status undergoing chemotherapy or on steroids; those with significant and unstable psychiatric illness or currently in prison; those with cognitive impairment or an inability to make decisions or those living in nursing homes; those aged < 21 or > 75 years; those who had bariatric surgery, including lap banding, gastric sleeve surgery, or cholecystectomy; those with >5% weight loss in the last 3 months prior to study enrollment; those who received antibiotics treatment within 6 weeks of enrollment; those who consumed lactuloseand/or dietary fibers for resolution of constipation; DM patients on stable doses of insulin sensitizers for at least 3 months; and those with any family history or past history of autoimmune disease, gastrointestinal cancers, inflammatory bowel disease, irritable bowel syndrome, and anxiety or depression.

All subjects provided written informed consent prior to their inclusion in the study. The enrolled subjects provided baseline stool and blood samples at Visit 1 and another postprandial blood sample for endotoxin analysis at Visit 2. They were required to complete a dietary questionnaire and undergo a methane breath test. The study was approved by the SingHealth Centralized Institutional Review Board (CIRB Ref: 2016/2090), Singapore, and was performed in accordance with the ethical standards of the Declaration of Helsinki (as revised in Brazil 2013). The study has been registered in clinicaltrials.gov (NCT03665961).

Clinical and laboratory tests. Baseline full blood count, renal function, liver function test, fasting glucose, glycated hemoglobin, lipid profile, ferritin, fasting insulin, and cholesterol profile were assayed at the Department of Laboratory Medicine, Changi General Hospital. IR was measured by the calculated homeostatic model assessment (HOMA) method (HOMA-IR = [insulin × glucose]/22.5). Only subjects who passed the screening tests were enrolled. The enrolled subjects provided a baseline stool sample and blood samples for cytokine and FibroTest analyses. The subjects' fat composition was analyzed using Tanita Body Fat Composition Analyser (Tanita, Japan).

The new International Diabetes Federation (IDF) definition was used to define the presence of a metabolic syndrome. Metabolic syndrome is defined as the presence of central obesity (waist circumference ≥ 90 cm in males or ≥ 80 cm in females) with at least two of the following factors: (i) triglyceride ≥ 150 mg/dL or specific treatment for this lipid abnormality, (ii) high-density lipoproteins (HDL) cholesterol < 40 mg/dL in males or <50 mg/dL in females or specific treatment for this lipid abnormality, (iii) systolic BP ≥ 130 or diastolic BP ≥ 85 mm Hg or treatment of previously diagnosed hypertension, and (iv) fasting plasma glucose ≥ 100 mg/dL or previously diagnosed type 2 DM.

Dietitian assessment. To evaluate baseline intake, each subject was advised by a dietitian with regard to completing a 3-day dietary record (3DDR) for two weekdays and one weekend. Subjects were shown examples of serving size for food from the four food groups and given guidance on their intake recording for each meal. The diet record form consisted of columns to note the meal type/time, type of food, amount, cooking methods, and place. Subjects were required to submit the food record for analysis of mean daily caloric and macronutrient intakes, which were calculated using the Dietplan6 software (Forestfield Software Ltd., Horsham, UK).

ELISA assays. Serum samples were assayed for a panel of inflammatory cytokines, namely, interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), and adiponectin (R&D Systems, Minneapolis, Canada). Associations between host inflammatory

responses, such as endotoxemia with central obesity, as well as the biomarker of NASH, cleaved cytokeratin 18 (Perviva, Sissach, Switzerland), were also examined. Endotoxemia was assessed by measuring levels of lipopolysaccharide binding protein (LBP) (MyBiosource, California, United States) and limulus amebocyte lysate (LAL) (Hycult Biotech, Uden, Netherlands) in the serum. LBP is a soluble acute-phase protein that binds to bacterial LPS to elicit immune responses. LAL assay measures bacterial endotoxin, a by-product of Gram-negative bacteria. All biomarkers were assayed using commercially available ELISA kits according to manufacturer's instructions.

Extraction of microbial DNA from stool samples. Aliquots of fresh stool samples (approximately 3 g) were collected into stool collection containers, frozen immediately, and/or transported with ice packs to the laboratory within 4 h of collection. The samples were stored at -80°C until DNA extraction. Microbial DNA was extracted from stool samples using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). DNA quality was assessed using gel electrophoresis and nanodrop.

16S rRNA amplification and sequencing. A region on the 16S rRNA spanning the V3–V6 domains was amplified from total DNA as previously described.[21] Amplicons were sheared using the Covaris LE220 sonicator (Covaris, Inc., USA) and built into sequencing libraries using GeneRead DNA Library I Core Kit (Qiagen) according to the manufacturer's protocol. DNA libraries were multiplexed by 96 indices, pooled, and sequenced on the Illumina HiSeq 2500 using paired-end (2x76bp) sequencing. Sequencing reads were demultiplexed (Illumina bcl2fastq 2.17.1.14 software) and filtered (PF = 0) before conversion to FASTQ format. Reads were trimmed by removal of trailing bases with a quality score ≤ 2. Read pairs containing reads shorter than 60 bp were also removed.

Reconstruction and classification of 16S rRNA amplicon sequences. Paired-end short DNA sequencing reads were used to reconstruct long 16S rRNA amplicons using the Expectation Maximisation Iterative Reconstruction of Genes from the Environment (EMIRGE) algorithm.[22] All high-quality reads were input to capture the 16S diversity whilst decreasing the computational requirements;[23] updated tools can be found at https://github.com/CSB5/GERMS_16S_pipeline. The EMIRGE amplicon uses 16S rRNA sequences on the SILVA database for template-guided assembly of reconstructions, followed by an expectation-maximization algorithm to iterate, align, and assign reads to candidate 16S sequences.[22] This iterative mapping of paired-end reads also prevents chimeric sequences from mapping. Reconstructed sequences with at least 99% sequence similarity were collapsed into operational taxonomic units (OTUs), and Graphmap[24] was used to map these OTUs to the Greengenes global rRNA database (dated May 2013; greengenes/13_5/99_otus.fasta).[25]

OTUs were called at various levels of identity (species, genus, family etc.) dependent on the sequence similarity to the Greengenes database as defined by Yarza and colleagues.[26] Hits below a predefined sequence similarity (97% at the species level, 94.5% at the genus level, 86.5% at the family level, and 75% at the phylum level) were not considered for classification and removed. The EMIRGE assigned abundance estimates to reconstructed sequences. The relative abundance of OTUs was generated for each sample and converted to relative abundances at the phylum, family, genus, and species levels. OTUs mapping to the database with a higher-sequence similarity were called to a more precise level of identity as a result.

Table 1 Baseline characteristics of subjects

Parameter	No central obesity (N = 13)	Central obesity (N = 22)	P value
Age (years)	30.3 ± 8.0	45.6 ± 14.1	0.03†
Male/female	6/7	17/5	0.03†
Ethnicity			0.21
Chinese	4	10	
Malay	6	4	
Indian	3	8	
BMI (kg/m^2)	21.6	29.2	0.002†
Body fat (%)	23.7 ± 7.0%	29.9 ± 11.3%	0.005†
Type 2 DM	0	8	<0.001†
Systolic blood pressure (mmHg)	113 ± 12	127 ± 12	0.94
Diastolic blood pressure (mmHg)	75 ± 10	82 ± 6	0.09
Alanine aminotransferase	19 ± 11	28 ± 20	0.19
Aspartate aminotransferase	25 ± 8	28 ± 12	0.24
Fasting glucose	4.6 ± 0.3	5.8 ± 1.4	<0.001†
Triglyceride	0.81 ± 0.30	1.08 ± 0.32	0.71
HDL	1.49 ± 0.30	1.41 ± 0.30	0.54
LDL	2.72 ± 0.78	3.19 ± 0.62	0.54
HOMA-IR	0.98 ± 0.53	2.68 ± 1.84	0.01†

†Statistical difference at 5% level of significance.
HDL, high-density lipoproteins; HOMA-IR, homeostatic model assessment-insulin resistance; LDL, Low-density lipoproteins.

Data visualization and statistical analyses. Differences in bacterial communities among the various test groups were assessed using distance-based redundancy analysis (dbRDA), based on Bray-Curtis distances, with the capscale function of the vegan package in R. Permutational Multivariate Analysis of Variance (PERMANOVA) applied based on 999 permutations to assess if microbial community composition differences were significant between every two test groups. The envfit function of the vegan package was used to investigate and visualize correlations among different bacteria influencing their overall microbial structure. Statistical significance of relative abundance between two groups was assessed using the Mann–Whitney U-test. Shannon alpha diversity was obtained for each sample and plotted on R using the package ggplot2.

Results

A total of 35 study subjects were recruited, comprising 14 Chinese, 10 Malay, and 11 Indian patients. There were 23 male and 12 female subjects. The median age was 39 years (range: 22–70 years). Five of the subjects were deemed to have a metabolic syndrome

Table 2 Dietary component analysis

	Total subjects (N = 35)				
	RDA (target)	Grouping (%)	Number	%	Reference
Protein	10–15%	≤15%	11	31	[27]
		>15%	24	69	
Fat	25–30%	≤30%	3	9	[27]
		>30%	32	91	
Carbohydrate	55–65%	<55%	30	86	[27]
		≥55%	5	14	
Sugar	≤10% of daily energy	<10%	14	40	[27]
		>10%	21	60	
Saturated fat	≤10% of daily energy	<10%	14	40	[27]
		>10%	29	83	
Fiber	25 g	<25 g	35	100	[28]
		>25 g	0	0	
Monounsaturated fat	—	<15%	32	91.4	[29]
		>15%	3	8.6	
Polyunsaturated fat	—	<5%	8	23	[29]
		>5%	27	77	

RDA, recommended dietary allowance.

according to the new IDF definition; nine subjects had BMI > 28 kg/m^2, and two of them had type 2 DM. Among the enrolled subjects, 22 had central obesity according to the WHO definition. The demographics and baseline characteristics of the study subjects, including clinical biochemistry profiles, are shown in Table 1.

Table 2 tabulates the dietary component analysis based on recommended dietary allowance (RDA). Based on the RDA, the percentages of subjects who met the cut-off values within the obesity and nonobesity groups are shown in Figure 1. Subjects from both groups had high proportions of high fat (>30%), high saturated fat (>10%), and low fiber (<10 g) intake. There were no significant differences in the intake pattern of the dietary components with the exception of sugars, for which a higher percentage of the obese group consumed >10% compared to the nonobese group (P = 0.049).

From the ELISA-based biomarker analysis, there were no significant associations between all the tested inflammatory cytokines, endotoxins, and biomarkers of NASH. There was a trend of raised IL-6 in the central obesity group (1.32 ± 0.86 pg./mL) compared to the nonobese group (0.96 ± 0.78 pg./mL); however, the difference was not statistically significant (P = 0.21).

Analysis of the alpha diversity indices did not demonstrate significant differences in species richness between obesity and ethnic status (Fig. 2a). While no distinct clustering was apparent based on BMI status, we observed overrepresentation of higher BMI (>23) subjects in the space matrix, which indicates a fiber-

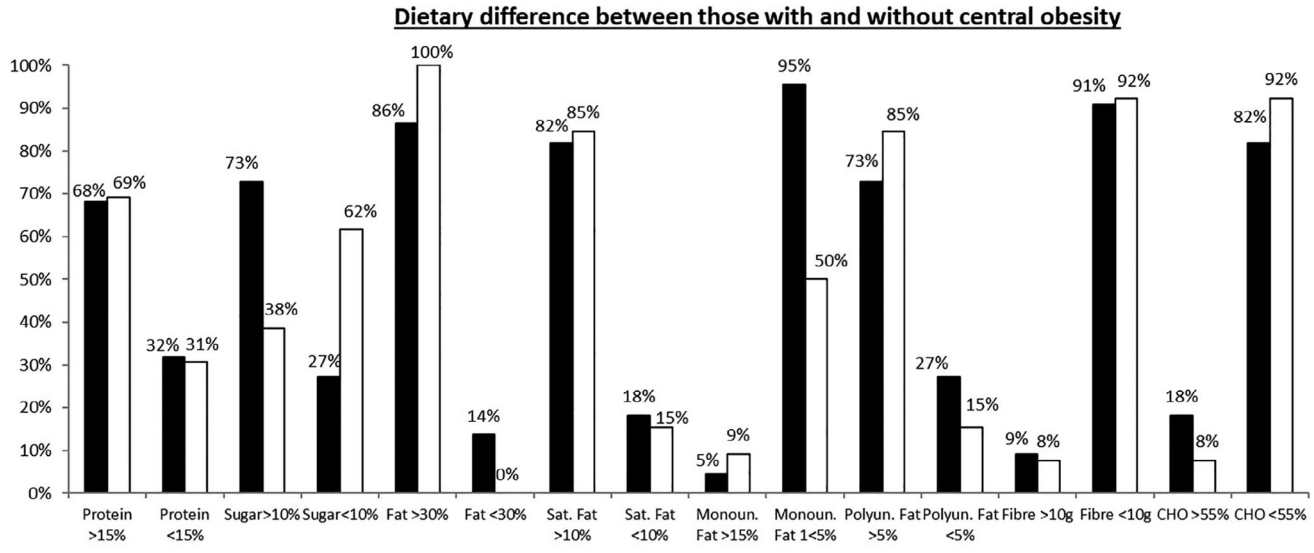

Figure 1 Percentages of subjects who met the recommended dietary allowance (RDA) cut-off within the obesity and nonobesity groups. (■) Yes central obesity, (□) no central obesity.

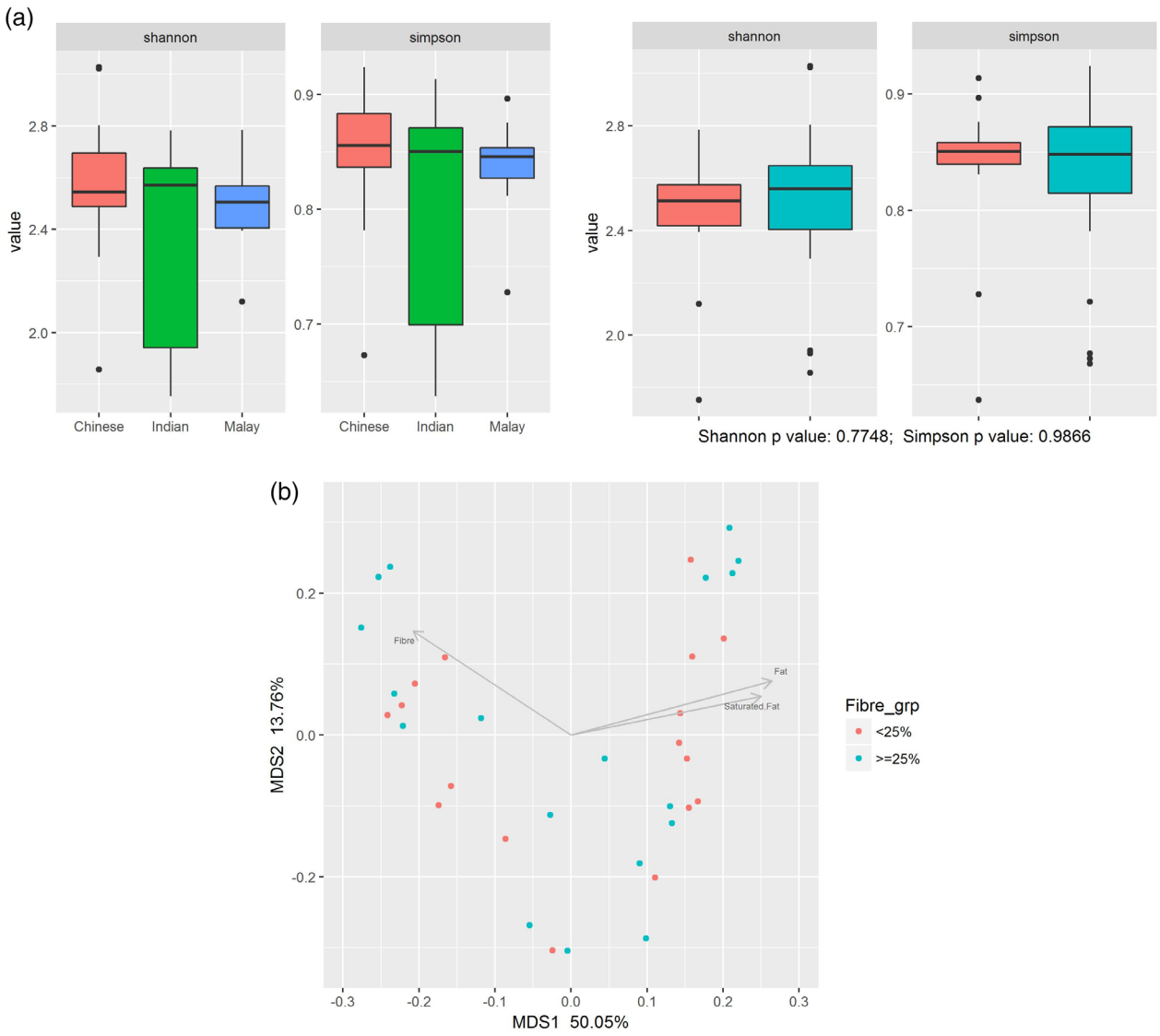

Figure 2 (a) Box-whisker plots of the alpha diversity indices for ethnicity (left) and obese "1"/nonobese "0" (right) groups. (■) Chinese, (■) Indian, (■) Malay, (■) 0, (■) 1. (b) Principal coordinate analysis (PCoA) based on microbial relative abundance on body mass index (BMI) factor. Each point represents an individual. Arrows indicate additional significant factors (fiber, fat, unsaturated fat), and the length of the arrow correlates with the magnitude of each factor. (●) <23, (●) >23.

driven microbial profile from the principle coordinate analysis (PCoA) (Fig. 2b). We calculated the significant vectors/arrows that were a projected coordinate system from variables—in this case, all the metadata including protein, fat, carbohydrate, sugar, saturated fat, fiber, monounsaturated fat, polyunsaturated fat, fructose, and vitamin E. However, only fiber, fat, and saturated fat were plotted as significantly related to BMI < or > than 23. They appeared to act in opposition, which is interesting because diets high in fiber were known to be lower in fats.[30] The PCoA plot showing Bray-Curtis dissimilarities at the genus level of the gut microbiome between the BMI < and > 23 groups showed no apparent compositional differences (Figure S1, Supporting information).

Relative abundances of *Anaerofilum* (P = 0.02), *Gemellaceae* (P = 0.02), *Streptococcaceae* (P = 0.03), and *Rikenellaceae* (P = 0.04) were significantly lower in the obese group. The *Prevotella* to *Bacteroidetes* ratio was not significantly different between obese and nonobese groups (Fig. 3). The heatmap displaying composition difference of signature taxa at the genus level is shown in Figure S2. *Catenibacterium* and *Succinivibrio* were found to correlate positively with TNF-α, while *Bilophila* correlated negatively with TNF-α.

Discussion

This study evaluated the gut microbiome pattern and cytokine profiles of 35 Asian subjects, comprising a male to female ratio of 2:1 and an almost equal distribution of the three major local ethnic groups, Chinese, Malay, and Indian. The parameters analyzed included the association of alpha diversity index, beta diversity index, signature taxa identification, and

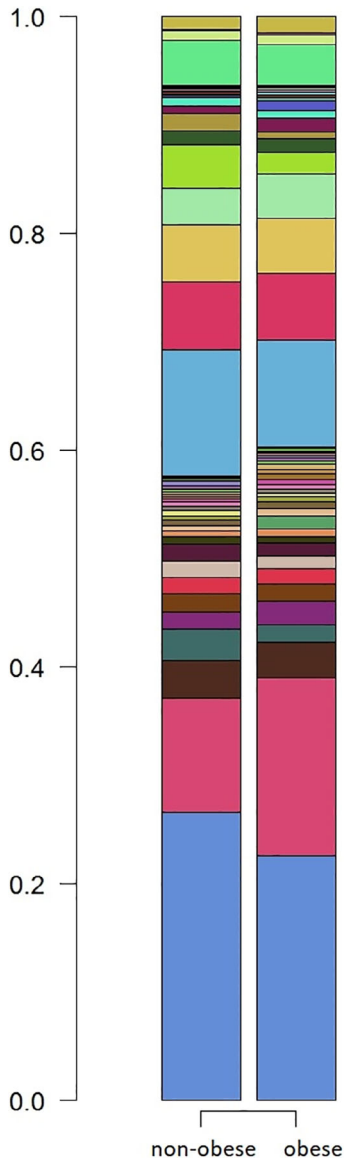

Figure 3 Operational taxonomic unit (OTU) profiles of nonobese and obese groups. (■) Low abundance, (■) o.YS2, (■) o.Bacteroidales, (■) o. Clostridials, (■) f.Odoribacteraceae, (■) f.Erysipelotrichaceae, (■) f.Christensenellaceae, (■) f.Coriobacteriaceae, (■) f.Alcaligenaceae, (■) f. Clostridiaceae, (■) f.Paraprevotellaceae, (■) f.S24.7, (■) f.Porphyromonadaceae, (■) f.Veillonellaceae, (■) f.Barnesiellaceae, (■) f. Enterobacteriaceae, (■) f.Rikenellaceae, (■) f.Prevotellaceae, (■) f. Lachnospiraceae, (■) f.Bacteroidaceae, (■) f.Ruminococcaceae, (■) g. Catenibacterium, (■) g.Fusobacterium, (■) g.Collinsella, (■) g.Clostridium, (■) g.Veillonella, (■) g. Odoribater, (■) g. Bilophila, (■) g. Butyricimonas, (■) g.Megamonas, (■) g.Paraprevotella, (■) g.Klebsiella, (■) g.Mitsuokella, (■) g.Megasphaera, (■) g.Blautia, (■) g.Dorea, (■) g. Bifidobacterium, (■) g.Dialister, (■) g.Coprococcus, (■) g.Succinivibrio, (■) g.Acidaminococcus, (■) g.Roseburia, (■) g.Oscillospira, (■) g.Ruminococcus, (■) g.Phascolarctobacterium, (■) g.Lachnospira, (■) g. Sutterella, (■) g.Faecalibacterium, (■) g.Parabacteroides, (■) g. Prevotella, (■) g.Bacteroides.

Prevotella/Bacteriodetes ratio with central obesity. In addition, associations of central obesity with inflammatory cytokines, endotoxins, and biomarkers of NASH were examined.

The lack of significant associations between the inflammatory cytokines, endotoxins, and biomarkers of NASH with central obesity is in contrast to previous reports, which demonstrated a correlation between these pro-inflammatory markers and obesity. Roth *et al*. found significant correlations between adiponectin and several mediators of inflammation (IL-1β, IL-6, and IL-8 and tumor necrosis factor-α).[31] Terra *et al*. reported that circulating visfatin levels were positively related to IL-6 and C-reactive protein levels in a cohort of morbidly obese women.[32] Furthermore, the mean serum concentrations of adiponectin, TNF-α, and leptin were shown to be different in NASH patients compared to healthy controls.[27] Our observations could possibly be due to the small sample size and obesity without significant pathologies in the study population. The list of assayed biomarkers known to be raised in association with obesity is also not exhaustive, and many other potential candidate analytes, such as leptin, resistin, adipokines, IL-10, and visfatin, have not been included in this study.

The dietary survey showed a similar intake of dietary components, including high fat, high saturated fat, and low fiber intake, in both obesity and nonobesity groups, even though both groups had morphometrix differences. These results were similar to the National Nutrition Survey 2010,[33] implying that the dietary consumption of the study subjects is representative of the general population in Singapore. Overall, the cohort shared a relatively similar baseline dietary composition characterized by very low fiber and high fat and protein intake. The findings also demonstrated that the proportion of fiber and fat/saturated fat diets was not the key determinants of central obesity; rather, a higher consumption of sugars might lead to obesity, although this was only marginally significant. It is of note that the dietary patterns of subjects were based on self-recording, which was dependent on their individual level of understanding and diligence in completing the survey, and thus might not be the most accurate reflection of the actual dietary intake. Nonetheless, the 3-day dietary survey has been validated in scientific studies and has been demonstrated to be acceptable as dietary assessment tools.[34]

The observation of lower relative abundances of *Anaerofilum*, *Gemellaceae*, *Streptococcaceae*, and *Rikenellaceae* in the obese group is consistent with the report by Del Chierico *et al*., which demonstrated higher levels of *Rikenellaceae* in individuals of average weight compared to overweight subjects.[35] However, contrary to a previous report,[36] the relative abundance of *Streptococcaceae* was higher in mice with NASH on a high-fat diet. It is tempting to speculate that the contents of the diet influenced the outcome of the gut microbiome, that is, higher consumption of sugar is linked to lower relative abundances of *Anaerofilum*, *Gemellaceae*, *Streptococcaceae*, and *Rikenellaceae* in the obese group in this study. However, we cannot disregard or uncouple the effect of the host's immune system as evident from the many publications reviewed recently by Freidman.[37] Moreover, further experiments, such as validation using quantitative polymerase chain reactions, are required to confirm the study findings.

We acknowledge that there are other limitations associated with the study. The relatively small sample size of this study was

not powered to detect subtle differences in microbial diversity in the various subgroups. In addition, the baseline microbiome variability was not determined as the study did not sample multiple fecal samples for comparison. It is also worth noting that there were statistically significant ($P = 0.03$) differences in gender and age between the obese and nonobese, groups which might be confounding factors influencing the human gut microbiome. Moreover, microbiome profiling provides no indication on the actual functionality and consequence on the individual. Further functional and clinical studies are required to elucidate the mechanistic pathophysiology associated with microbiome differences.

In spite of these limitations, to the best of our knowledge, this represents the first study to examine the association of the gut microbiome with central obesity in the Asian population, as well as the intricate relationship with inflammatory biomarkers. The knowledge gained from this study lays the foundation for future larger-scale studies using shotgun metagenomics and bacterial metabolites to examine the bacterial dysbiosis in functional analysis.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's website:

Figure S1 PCoA plot showing Bray-Curtis dissimilarities at genus level of the gut microbiome between the body mass index (BMI) < and >23 groups.

Figure S2 Heatmap showing composition difference of signature taxa at genus level for the various analytes.