J. Microbiol. Biotechnol. (2016), 26(10), 1723–1735
http://dx.doi.org/10.4014/jmb.1603.03057

jmb

Microbial Community of Healthy Thai Vegetarians and Non-Vegetarians, Their Core Gut Microbiota, and Pathogen Risk

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Received: March 24, 2016
Revised: June 7, 2016
Accepted: July 1, 2016

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pISSN 1017-7825, eISSN 1738-8872

Pyrosequencing analysis of intestinal microflora from healthy Thai vegetarians and non-vegetarians exhibited 893 OTUs covering 189 species. The strong species indicators of vegetarians and non-vegetarians were *Prevotella copri* and *Bacteroides vulgatus* as well as bacteria close to *Escherichia hermanii* with % relative abundance of 16.9 and 4.5−4.7, respectively. Core gut microbiota of the vegetarian and non-vegetarian groups consisted of 11 and 20 different bacterial species, respectively, belonging to Actinobacteria, Firmicutes, and Proteobacteria commonly found in both groups. Two species, *Faecalibacterium prausnitzii* and *Gemmiger formicilis*, had a prevalence of 100% in both groups. Three species, *Clostridium nexile*, *Eubacterium eligens*, and *P. copri*, showed up in most vegetarians, whereas more diversity of *Collinsella aerofaciens*, *Ruminococcus torques*, various species of *Bacteroides*, *Parabacteroides*, *Escherichia*, and different species of *Clostridium* and *Eubacterium* were found in most non-vegetarians. Considering the correlation of personal characters, consumption behavior, and microbial groups, the age of non-vegetarians showed a strong positive correlation coefficient of 0.54 ($p = 0.001$) to *Bacteroides uniformis* but exhibited a moderate one to *Alistipes finegoldii* and *B. vulgatus*. Only a positive moderate correlation of body mass index and *Parabacteroides distasonis* appeared. Based on the significant abundance of potential pathogens, the microbiota of the non-vegetarian group showed an abundance of potential pathogen varieties of *Bilophila wadsworthia*, *Escherichia coli*, and *E. hermannii*, whereas that of the vegetarian group served for only *Klebsiella pneumoniae*. These results implied that the microbiota of vegetarians with high abundance of *P. copri* and low potential pathogen variety would be a way to maintain good health in Thais.

Keywords: Fecal microbiota, pyrosequencing, core gut microbiota, *Prevotella*, pathogen risk, Enterobacteriaceae

Introduction

In the last decade, many researchers worldwide have been interested in the human microbiota, especially gut microbiotas, which are the largest microbial community. Various factors such as diets [8, 12, 14, 20, 23, 50], age [30, 40, 52, 53], and diseases [9, 18, 29, 51] were reported for their effects on microbiota changes of their hosts. Diet is an important factor that has a close GI tract microbiota relationship. People who consumed high-fiber diets had a lower risk of cancer than those who had a high-meat diet [10, 36]. Such diets consisting of either plant materials or animal products as a main part may influence an individual's health. It was found that when diets change, the gut microbiota also changes; for example, research has been undertaken with people who consumed different diets, especially vegetarians and omnivores, in Africa [14], India [23], Japan [20], Slovenia [31], Thailand [45], and the United State of America [50].

In our previous study [45], fecal samples from 6 non-vegetarians and 7 vegetarians were investigated for their gut microbiota using polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE). Only 37 bands from 186 bands were identified as representative of 11 species: *Bacteroides thetaiotaomicron*, *B. ovatus*, *B. uniformis*, *B. vulgatus*, *Clostridium colicanis*, *Eubacterium eligenes*, *E. rectale*, *Faecalibacterium prausnitzii*, *Megamonas funiformis*, *Prevotella copri*, and *Roseburia intestinalis*, with ≥97% identity. They mainly belonged to the groups of *Bacteroides*, *Prevotella*, *Clostridium*, and *F. prausnitzii*. Although the dendogram from the PCR-DGGE profile could divide the DNA pattern into vegetarian and non-vegetarian groups, there were still many DNA bands that could not be identified. To gain more information on the microbial community of both groups who consumed different components in their diets, high-throughput pyrosequencing was used to investigate the gastrointestinal tract microbiota of Thai vegetarians and non-vegetarians with higher candidate numbers in this work. The correlations among gut microbiota, personal characters, consumption behavior, and the risk of pathogens were also considered.

Materials and Methods

Fecal Samples

Total fecal samples were obtained from 36 healthy vegetarians and 36 healthy non-vegetarians. The vegetarian volunteers consisted of ovo-lacto vegetarians, lacto-vegetarians, an ovo-vegetarian, and vegans. They all had been vegetarians for at least 3 years before participating in this study. All the subjects had regular bowel habits, including no change of defecation frequency, no history of gastrointestinal disease, such as gastritis, peptic ulcers, gastric cancer, colorectal cancer, or inflammatory bowel disease, no diarrhea in the month preceding the sampling, and no family history of colorectal cancer. None had received any antibiotic treatment within at least one month prior to this study. A stool sampling kit consisting of a sample collection tube, cotton swabs, and sterile tissue paper together with a questionnaire about each individual's consumption behavior and consent form were given to each subject. The study protocol and consent documents were approved by the Institute for the Development of Human Research Protections (IHRP) under ethics approval No. IHRP 311.

DNA Extraction

The total bacterial genomic DNA from each sample was extracted from 50 mg of each feces sample as described previously [45]. The DNA was eluted with sterilized pure water and kept at −20°C until use.

Preparation of Samples for Pyrosequencing Process

The 16S rDNA of bacterial DNA was amplified with a V6-V8 region specific primer set tagged with the barcode sequence, Q-968F-# (5′CWSWSWWSH WAC GCG ARG AAC CTT ACC3′) and Q-1390R-# (5′CWSWSWWSHTGA CGG GCG GTG WGT AC3′), where # is the serial number of the barcode tags for each treatment that contained nine different nucleotides at the 5′ end designed by Nakayama [37]. Each genomic DNA sample of approximately 10–100 ng was used as the template in 50 µl of PCR. For each sample, a PCR mixture containing 1× Ex *Taq* buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1.5 mM $MgCl_2$), 0.2 mM of each dNTP, 0.2 µM of each primer, and 1.25 U of TaKaRa Ex *Taq* HS (Takara Bio, Japan) was prepared. The PCR conditions were as follows: one cycle at 98°C for 2.5 min; 20 cycles at 98°C for 15 sec, 54°C for 30 sec, and 72°C for 20 sec; and a final elongation at 72°C for 5 min. The amplicons were purified using a QIAquick 96 PCR purification kit from Qiagen (Germany) according to the manufacturer's protocols. The purified PCR products were determined for the DNA concentration using a NanoDrop ND-1000 spectrophotometer. Approximately 100 ng of each purified amplicon from each sample was pooled and purified by ethanol precipitation prior to the pyrosequencing process [38].

The pyrosequencing process was performed according to the manufacturer's protocol (454 Life Sciences, Roche, the Netherlands), where the amplicon mixture was applied using emulsion PCR (emPCR) with a GS FLX Titanium LV emPCR kit (Lib-L) v2. By serial dilution, each amplicon fragment obtained was amplified on a special bead (one fragment per bead) and then loaded and fixed onto a GS FLX Titanium Pico Titer Plate with dividers separating the reaction chambers. The pyrosequencing was processed using an FLX Genome Sequencer (454 Life Sciences) with a GS FLX Titanium Sequencing Kit XLR70 according to the manufacturer's protocol.

Pyrosequencing Data Analysis

The obtained 454 batch data were sorted using the QIIME 1.7.0 software package to acquire each sample batch. Then, the multiplex reads were attributed to a split_library.py script (http://qiime.org/scripts/split_libraries.html) based on their barcode sequences to generate each sample sequence data batch. The parameters used in this script were performed according to Nakayama *et al.* [38] as follows: 1 (minimum sequence length) = 408, e (maximum number of errors in barcode) = 0, reverse primer mismatches = 3, a (maximum number of doubtful bases) = 3, and

L (maximum sequence length) = 500. In this step, any low-quality or doubtful reads were removed based on the characteristics of each sequence.

The total reads obtained from a set of split_library.py sequences were further filtered for their qualities to perform noisy sequence removal, chimera checking, and operation taxonomic unit (OTU) clustering via USEARCH v5.2.236 in QIIME (http://qiime.org/tutorials/usearch_quality_filter.html), using the command of "pick_otus.py -i seq.fna -m usearch -f gold.fa -o usearch_qf_result/ --word_length 64" to obtain OTU IDs that were further analyzed by the pick_rep_set.py script (http://qiime.org/scripts/pick_rep_set.html) to identify the species of each OTU ID. The phylogenetic composition was determined using the QIIME assign_taxonomy.py script (http://qiime.org/scripts/assign_taxonomy.html?highlight=assign_taxonomy) against the Greengenes 13_5 database. Finally, OTU tables were generated using the command "make_otu_table.py and convert_biom.py.scripts" (http://qiime.org/scripts/make_otu_table.html?highlight=make_otu_table). Chao1 and Shannon index values were calculated by alpha_diversity.py -i otu_table.biom -chao1 -o adiv_chao1.txt and alpha_diversity.py -i otu_table.biom -m shannon.txt, respectively.

Taxonomic Analysis

Each OTU sequence was classified into bacterial population data from the phylum to genus (hierarchical level) by the RDP classifier at the RDP website (http://rdp.cme.msu.edu/classifier/classifier.jsp). The confidence threshold for the RDP classifier was set at 80%. The identification at species level was run on RDP seqmatch (http://rdp.cme.msu.edu/seqmatch/seqmatch.jsp), which displayed the 20 closest 16S rDNA of cultured strains. After the seqmatch process, the seqmatch Q400 algorithm was used to convert the result of each sample from RDP seqmatch to the species level of the population [34]. The species found in each sample showed the best match on seqmatch Q400, which was assigned to the query sequence. If they revealed more than two species with the same best score, the one with the highest count on the top 20 lists was selected. The criteria used to identify species was cut off at ≥0.90 S_ab score. The relative abundance of each taxon was determined by dividing the assigned read counts by total read counts [38].

Statistical Analysis

Statistical analysis was performed using SPSS statistics software ver. 18. A normality test was performed using the one-sample Kolmogorov-Smirnov test to determine their distribution. For normally distributed data, differences between sample groups were compared using one-way ANOVA, whereas the Mann-Whitney U test was used for non-normally distributed data. Statistical significance was tested at a p value less than 0.05.

Principal component analysis (PCA) was determined using the Multi-Variate Statistical Package ver. 3.1. The first two principal components with high values in PCA were selected to demonstrate the PCA plot as the x-axis and y-axis. An arrow indicated a close relation of key bacterial species to that arrow axis.

Correlations between bacterial species and various factors were calculated using the bivariate correlation function of the SPSS statistical software. For normally distributed data, differences between sample groups were compared using a Pearson test, whereas the Mann-Spearman test was used for non-normally distributed data. Statistical significance was tested at a p value less than 0.05.

Results

Characterization of Thai Subjects

Fecal samples were randomly collected from 36 non-vegetarians aged between 41 and 78 years and from 36 vegetarians aged between 40 and 61 years. The average age of non-vegetarians and vegetarians was 51.8 ± 8.1 and 50.9 ± 5.9 years, respectively, showing no significant difference. All the non-vegetarians consumed red meat (only pork), white meat such as fish and chicken, and eggs (9 ± 4 eggs/month on average), plus only 75% of this group consumed yoghurt and milk (8 cups of yoghurt per month and 12 glasses of milk per month on average). Most vegetarians were living and working in a community called Santi-Asoke, except the subjects V1, V2, and V36. All vegetarian volunteers were grouped as ovo-lacto-vegetarians (n = 4), lacto-vegetarians (n = 28), ovo-vegetarian (n = 1), or vegans (n = 3). They had been vegetarians for 3 to 35 years (21.2 ± 9.0 years on average). Among the ovo-lacto-vegetarians and lacto-vegetarians, 16 subjects drank milk (4 glasses/month on average), whereas 30 subjects consumed yoghurt (7.5 cups/month on average). Only five vegetarians consumed eggs (1 egg/month on average). The vegetarians and non-vegetarians all consumed Thai fruit on a daily basis. The body mass index (BMI) of the vegetarians and non-vegetarians was 21.57 ± 2.67 and 24.74 ± 3.52 kg/m^2, respectively, which had significant difference ($p < 0.01$) due to the diet type supported by the work done by Tonstad *et al*. [47] and Zhang *et al*. [54]. So far, several factors are known to affect microbiota change [8, 12, 14, 20, 23, 50]. Therefore, BMI change might be a factor affecting the microbiota of these subjects.

Pyrosequencing Analysis of Gut Microbiota of Thai Vegetarians and Non-Vegetarians

The microbial diversity of Thai gut microbiota was evaluated for species richness by Chao1 and for both richness and evenness by the Shannon diversity index from all OTU data for each sample (Fig. 1). The total number of Chao1 richness in the non-vegetarian group (266.34 ±

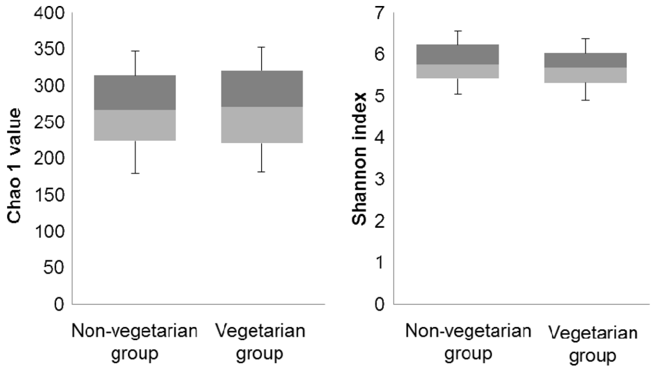

Fig. 1. Boxplots of the microbial diversity of Thai non-vegetarians and vegetarians for species richness by Chao1 and for both richness and evenness by the Shannon diversity index from the OTU data for each sample.

The gray box represents the ratio of 25th to 50th percentile, and the dark gray box represents the ratio of 50th to 75th percentile.

64.78) was slightly lower than vegetarians (270.83 ± 72.11) without significance ($p = 0.78$). The diversities determined by the Shannon index of the non-vegetarian and vegetarian groups were 5.76 ± 0.70 and 5.67 ± 0.59, respectively, with no significant difference ($p = 0.58$).

Phylum Level

Of the total 273,419 non-chimera reads obtained from both sample groups of healthy Thai vegetarians and non-vegetarians, 134,800 reads belonged to vegetarians whereas 138,619 reads were from non-vegetarians. From these reads, 893 OTUs were obtained. The 663 OTUs obtained were overlapped by both subject groups, while 99 and 131 OTUs belonged to only vegetarians and non-vegetarians, respectively. These OTUs from non-vegetarians belonged to nine phyla consisting of Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria, and others consisting of Cyanobacteria/Chloroplast, Elusimicrobia, Fusobacteria, Verrucomicrobia, and TM7, whereas the ones from the vegetarians belonged to similar phyla except for the phylum Elusimicrobia. The Firmicutes and Bacteroidetes were the two largest phyla found in the Thai gut samples (Fig. 2). The nine phyla contained representatives of 16 classes, 41 families, 92 genera, and 189 species, which implied diversity of the gut microbiota detected.

Family Level

The differences of gut microbiota between vegetarians and non-vegetarians had appeared at the family level. In total, 41 families were detected in Thai gut microbiota, where the different families found only in either non-vegetarian or vegetarian were Elusimicrobiaceae and Moraxellaceae or Clostridiales_Incertae Sedis XIII and Flavobacteriaceae, respectively. Of those families with >1% relative abundance found in both subject groups (Fig. 3), Prevotellaceae had a highly significant abundance in vegetarians (p = 2.89E-09), whereas Bacteroidaceae was significant in non-vegetarians (p = 2.35E-06). In addition, it was also found that the abundance of Verrucomicrobiaceae, Rikenellaceae, and Porphyromonaceae in the non-vegetarian group was significantly higher than in the vegetarian group by p = 5.77E-05, 3.83E-03, and 2.29E-02, respectively, whereas the rest showed no significant difference even

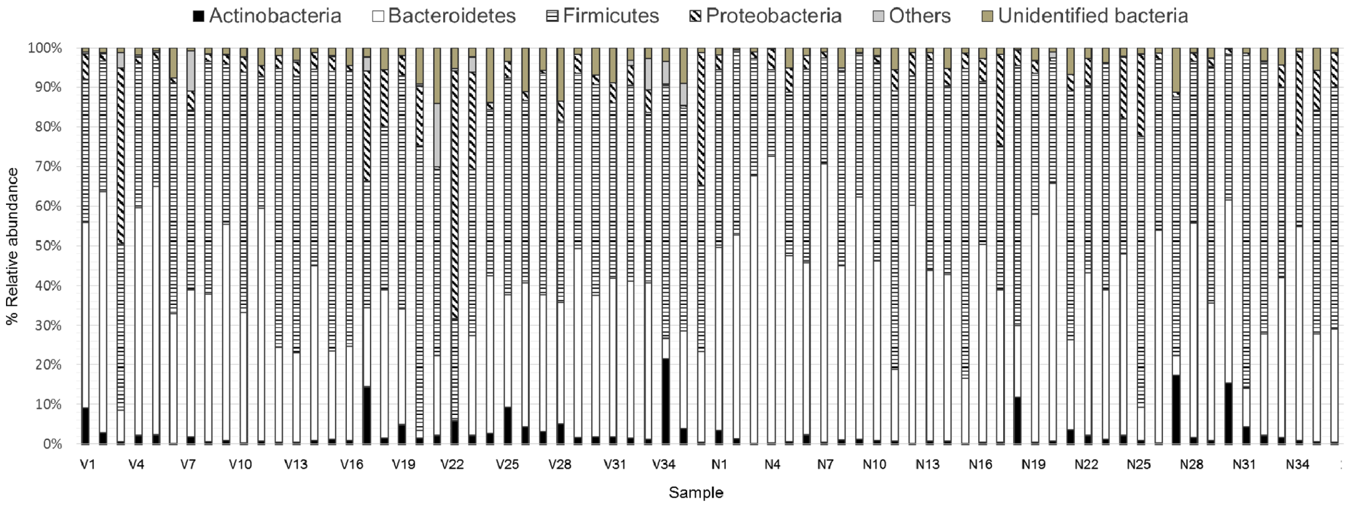

Fig. 2. Gut microbiota detected in both sample groups at the phylum level.

N represents non-vegetarian subjects, and V represents vegetarian volunteers. The number of either N or V is aligned in order of 1–36.

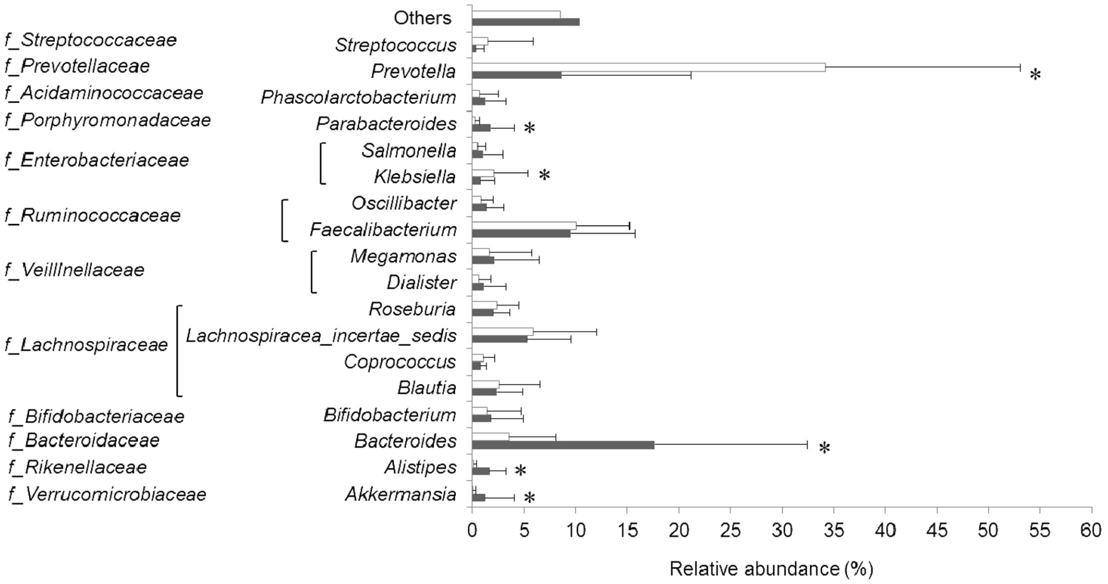

Fig. 3. Abundance of gut microbiota at the family and genus levels detected in both vegetarian and non-vegetarian groups.
Abundance of the families and genera with >1% relative abundance from the vegetarian group (white bar) and non-vegetarian group (dark gray bar). An asterisk indicates genera with significant difference ($p < 0.05$).

though the abundance level of Enterobacteriaceae, Lachnospiraceae, and Ruminococcaceae from both groups was high.

Genus Level

At the genus level, both high and low relative abundance levels were determined based on the relative abundance cut-off of 1%. There were 345 OTUs commonly found in both subject groups belonging to 80 genera. The genera with high relative abundance found in both subject groups are shown in Fig. 3. The highest relative abundance of 34.2% and 17.68% found in the vegetarian and non-vegetarian groups were *Prevotella* and *Bacteroides*, respectively. Only six genera showed a significant difference among the vegetarian and non-vegetarian groups. *Prevotella* and bacterial species close to *Klebsiella* were significantly higher in the vegetarian group, whereas *Akkermansia*, *Alistipes*, *Bacteroides*, and *Parabacteroides* were in the non-vegetarian group.

Faecalibacterium and *Lachnospiracea_incertae_sedis* belonging to the family Ruminococcaceae and Lachnospiraceae had high abundance levels of 10.36–10.63% and 5.3–5.86%, respectively; however, their abundance levels in the vegetarian and non-vegetarian groups were not significantly different. Some genera were only detected in either the vegetarian or non-vegetarian group. Four genera, *Acinetobacter*, *Bulleidia*, *Caldimonas*, and *Elusimicrobium*, were found in the vegetarian group, whereas the five genera *Acidaminococcus*, *Pediococcus*, *Peptoniphilus*, *Succinivibrio*, and *Turicibacter* appeared only in the non-vegetarian group.

Species Level

From species identification based on RDP seqmatch using a cut-off >0.90 S_ab score, a total of 173,518 reads were obtained from both sample groups. Of these, 80,883 reads were obtained from the vegetarian group providing 2,246.75 ± 638.01 reads on average per subject, while 92,635 reads were from the non-vegetarian group having 2,573.19 ± 788.42 reads on average per subject. Two hundred fifty-five and 264 OTUs belonged to the vegetarian and non-vegetarian groups, respectively. Based on the relative abundance at species level of 0.1% shown in Table 1, the highest abundant species of 10.63% and 16.9% detected from the non-vegetarian and vegetarian groups were *Faecalibacterium prausnitzii* and *Prevotella copri*, respectively. The relative abundance of *F. prausnitzii* in the vegetarian group was also high at 10.36%, whereas the other species in both subject groups were lower than 5%. The abundance of seven species of non-vegetarians was significantly higher than for the vegetarian group, whereas only two of the bacterial species close to *Klebsiella pneumoniae* and *P. copri* in the vegetarian group were significantly higher than in the non-vegetarian group. It was noticed that all five species of *Bacteroides* detected also showed a higher

Table 1. Relative abundance of bacterial species found in Thai non-vegetarians and vegetarians.

Species	Non-vegetarian group		Vegetarian group		*p* Value
	Relative abundance (%)	Prevalence (%)	Relative abundance (%)	Prevalence (%)	
Alistipes finegoldii	0.39	88.9	0.09[c]	61.1	ND
Alistipes putrednis	0.96	80.6	0.03[c]	27.8	ND
Bacteroides caccae	0.54	75.0	0.08[c]	36.1	ND
B. dorei	3.03[a]	77.8	0.56	63.9	<0.01
B. thethiotaomicron	0.40[a]	91.7	0.13	61.1	<0.01
B. uniformis	2.45[a]	91.7	0.40	81.1	<0.01
B. vulgatus	4.50[a]	91.7	1.27	88.9	0.02
Bilophila wadsworthia	0.16	86.1	0.01[c]	30.6	ND
Blautia wexlerae	0.81	94.4	1.04	97.2	0.81
Clostridium clostridioforme	0.41[a]	94.4	0.19	83.3	0.02
C. nexile	0.54	88.9	0.28	91.7	0.07
C. orbiscindens	0.27	91.7	0.03[c]	44.4	
Collinsella aerofaciens	0.95	94.4	0.64	88.9	0.29
Coprococcus eutactus	0.17	50.0	0.41	77.8	0.08
Dorea formicigenerans	0.20	86.1	0.14	83.3	0.14
Dorea longicatena	0.47	94.4	0.36	94.4	0.37
Escherichia coli	1.53	94.4	0.65	86.1	0.09
E. hermannii	4.70[a]	97.2	0.72	86.1	0.03
Eubacterium eligens	1.82	88.9	1.20	94.4	0.26
Eu. hadrum	0.78	91.7	0.59	80.6	0.59
Eu. rectale	2.14	91.7	3.84	91.7	0.13
Faecalibacterium prausnitzii	10.63	100.0	10.36	100.0	0.84
Gemmiger formicilis	1.33	100.0	0.84	100.0	0.14
Klebsiella pneumoniae	0.79	94.4	2.17[b]	91.7	0.03
Odoribacter splanchnicus	0.25	75.0	0.06[c]	44.4	ND
Parabacteroides distasonis	1.22[a]	100.0	0.17	80.6	<0.01
Prevotella copri	3.80	77.8	16.90[b]	94.4	<0.01
Raoultella ornithinolytica	0.28	77.8	0.62	86.1	0.06
Roseburia faecis	0.65	86.1	0.54	80.6	0.52
Roseburia hominis	0.35	75.0	0.32	75.0	0.85
Ruminococcus obeum	0.36	97.2	0.57	97.2	0.17
Rumincoccus torques	0.53	94.4	0.53	86.1	0.10
Streptococcus thermophiles	0.32	77.8	1.42	88.9	0.14

% relative abundance calculated by (the number of read of each species / total non-chimera read of the subject) × 100.

[a] Non-vegetarian group was significantly higher than the vegetarian group.

[b] Vegetarian group was significantly higher than the non-vegetarian group.

[c] Relative abundance was not reliable since it was lower than 0.1%.

ND, not determined.

significant abundance in non-vegetarians, which corresponded to previous work done by Ruengsomwong *et al*. [45].

When the % relative abundance of each species from each subject was analyzed by PCA as shown in Fig. 4, it was found that most of the subjects in the vegetarian group tended to fall into the x-axis-positive region, whereas the majority of the subjects in the non-vegetarian group were situated in the x-axis-negative region. The loading plot

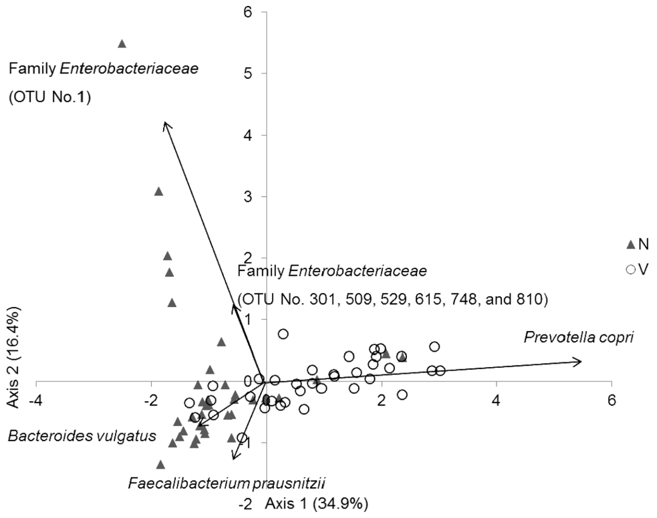

Fig. 4. PCA study of the abundant species from the vegetarian and non-vegetarian groups.

N, non-vegetarians (triangle); V, vegetarians (circle); axis-1 and -2 are the x and y axis, respectively.

representing the bacterial species showed that *P. copri* was closely aligned with the x-axis -positive group, which correlated to most of the vegetarian subjects. On the other hand, *Bacteroides vulgatus* and the family Enterobacteriaceae consisting of bacterial species close to *Escherichia hermannii* (OTU No. 1) and *E. coli* (OTU Nos. 301, 509, 529, 615, 748, and 810) were more closely aligned with the x-axis-negative group correlating to the majority of the subjects from the non-vegetarian group. These results implied that *P. copri* was the key species of vegetarians, whereas *Bacteroides vulgatus*, *E. coli,* and *E. hermanii* were the key species for non-vegetarians. Even though *F. prausnitzii* was abundant in both subject groups, it showed no significant difference as proposed by the species level.

Core Gut Microbiota of Vegetarians and Non-Vegetarians

The core gut microbiota in this study was defined as microorganisms found in all samples that had ≥90% prevalence. Twenty and 11 different bacterial species were found in non-vegetarians and vegetarians, respectively, as shown in Table 2, resulting in higher microbial diversity presented by non-vegetarians. Eight species (*Blautia wexlerae*, *Dorea longicatena*, *Eubacterium rectale*, *F. prausnitzii*, *Gemmiger formicilis*, *K. pneumoniae*, *Roseburia inulinivorans*, and *Ruminococcus obeum*) belonging to the three main phyla

Table 2. List of bacterial species of Thai vegetarians and non-vegetarians having ≥90% prevalence.

Non-vegetarians		Vegetarians	
Species	Prevalence (%)	Species	Prevalence (%)
Faecalibacterium prausnitzii	100.0	*Faecalibacterium prausnitzii*	100.0
Gemmiger formicilis	100.0	*Gemmiger formicilis*	100.0
Parabacteroides distasonis	100.0	*Roseburia inulinivorans*	97.2
Escherichia hermannii	97.2	*Blautia wexlerae*	97.2
Roseburia inulinivorans	97.2	*Ruminococcus obeum*	97.2
Ruminococcus obeum	97.2	*Prevotella copri*	94.4
Escherichia coli	94.4	*Eubacterium eligens*	94.4
Collinsella aerofaciens	94.4	*Dorea longicatena*	94.4
Blautia wexlerae	94.4	*Eubacterium rectale*	91.7
Klebsiella pneumoniae	94.4	*Klebsiella pneumoniae*	91.7
Ruminococcus torques	94.4	*Clostridium nexile*	91.7
Dorea longicatena	94.4		
Parabacteroides merdae	94.4		
Clostridium clostridioforme	94.4		
Bacteroides vulgatus	91.7		
Bacteroides uniformis	91.7		
Eubacterium rectale	91.7		
Eubacterium hadrum	91.7		
Bacteroides thethiotaomicron	91.7		
Clostridium orbiscindens	91.7		

of Actinobacteria, Firmicutes, and Proteobacteria, were commonly found in both the non-vegetarian and vegetarian groups. *Clostridium* and *Eubacterium* were found in both vegetarian and non-vegetarian groups but as different species. Two species (*F. prausnitzii* and *G. formicilis*) showed 100% relative prevalence in both subject groups, while an additional two and three species of *Parabacteroides* and *Bacteroides*, respectively, as well as *Escherichia* and *Ruminococcus torques* showed high prevalence only in the non-vegetarian group. When the % relative abundance is taken into account, *P. copri* showing a high relative abundance of 16.9% could be a strong indicator of a Thai vegetarian.

Correlation of Gut Microbiota, Personal Characters, and Consumption Behavior

The correlation of the abundance of all bacterial species from either vegetarians alone, non-vegetarians alone, or from all subjects and personal characters (BMI, age, time of being vegetarian) as well as consumption behavior (yoghurt and egg consumption) were investigated and determined as correlation coefficient (r) values and analyzed using the bivariate correlation function in the SPSS statistical software. Based on high, moderate, and weak correlation levels in the ranges 0.51−0.8, 0.31−0.5, and 0.1−0.30, respectively, only the age of the non-vegetarian group and the two species *Alistipes finegoldii* and *B. vulgatus* showed a moderate positive correlation efficient of 0.331 and 0.379, as presented in Figs. 5A and 5B, respectively, whereas *B. uniformis* did have a high correlation efficient of 0.54 ($p = 0.001$) (Fig. 5C). However, there was a negative, moderate correlation to *Roseburia faecis* (Fig. 5D).

In addition, the BMI and *Parabacteroides distasonis* and *Parabacteroides merdae* from all subjects showed moderate and weak positive correlations, as presented in Figs. 6A and 6B, respectively, whereas weak negative correlations occurred with regard to *P. copri* and *Raoultella ornithinotyca,* as shown in Figs. 6C and 6D, respectively.

To compare differences in microbiota within each type of vegetarian (lacto-vegetarians, ovo-lacto-vegetarians, ovo-vegetarian, and vegans), PCA was performed as showed in Fig. 7. The results showed no significant differences between the microbiotas in terms of genus and species of each type of vegetarian.

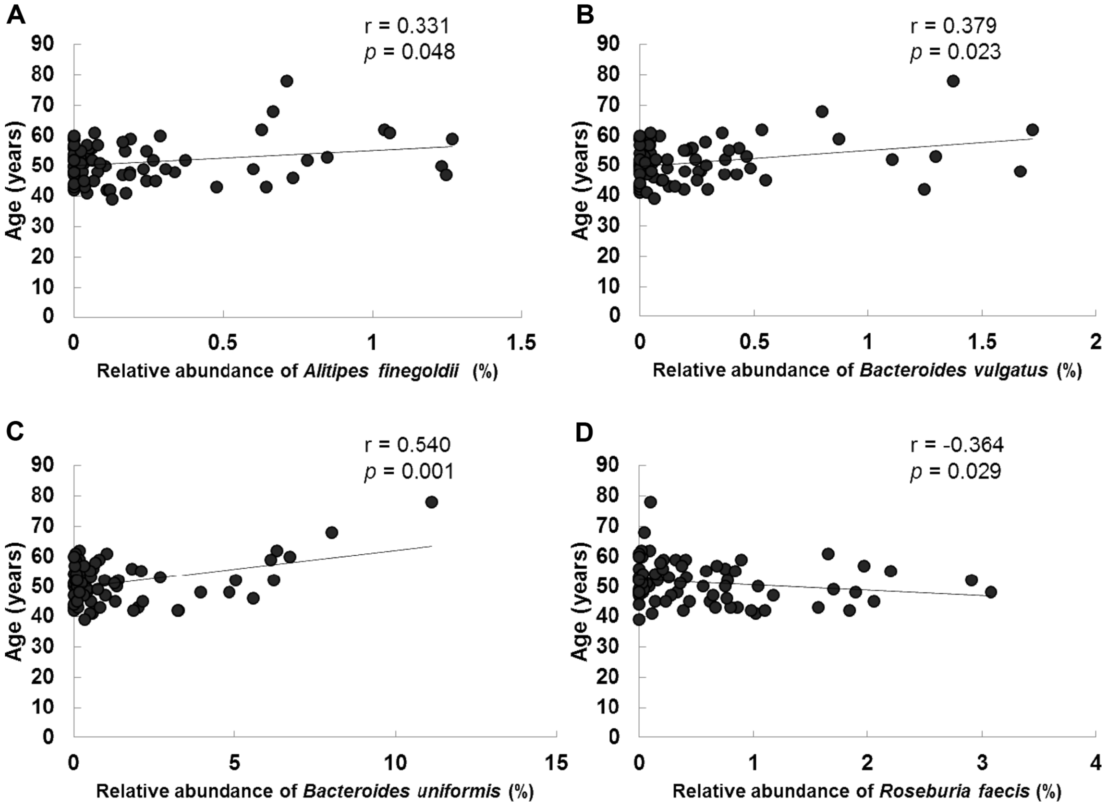

Fig. 5. Correlation between age of non-vegetarians and *Alistipes finegoldii* (**A**), *Bacteroides vulgatus* (**B**), *B. uniformis* (**C**), and *Roseburia faecis* (**D**).

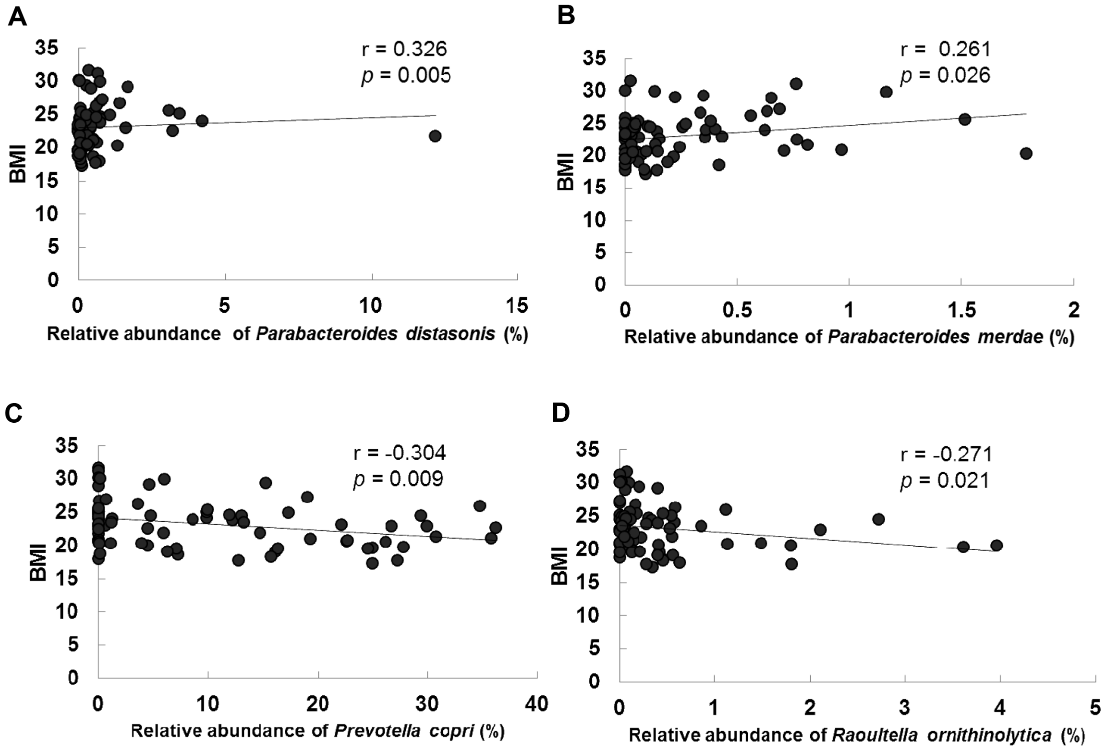

Fig. 6. Correlation of BMI and *Parabacteroides distasonis* (**A**), *Parabacteroides merdae* (**B**), *Prevotella copri* (**C**), and *Raoultella ornithinolytica* (**D**).

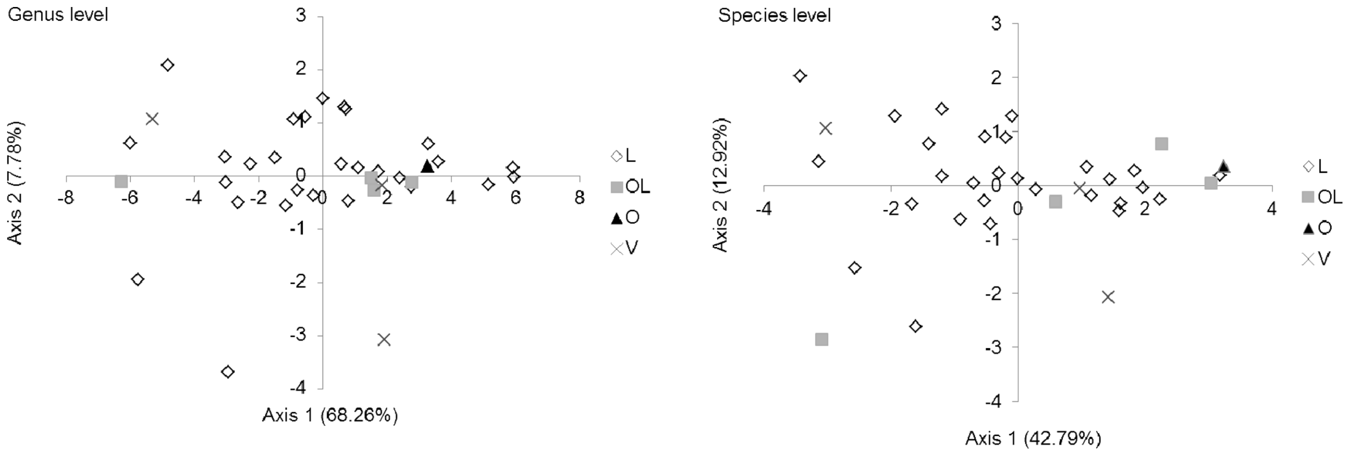

Fig. 7. PCA study of the intestinal microbiota of each type of vegetarian.
L, lacto-vegetarians (◇); OL, ovo-lacto-vegetarians (■); O, ovo-vegetarian (▲); V, vegans (×).

Opportunistic Pathogens Found in Vegetarians and Non-Vegetarians

Six species of bacterial pathogens were found in both subject groups, as shown in Table 3. Three bacterial species (*E. coli*, *E. hermannii*, and *K. pneumoniae*) had high % prevalence values of 94–97%. Only one bacterial species (*K. pneumoniae*) from the vegetarian group had a significantly higher % relative abundance than the one from the non-vegetarian group, and the relative abundance levels of *Bilophila wadsworthia* and *E. hermannii* from the non-vegetarian group were significantly higher than those in vegetarians.

Table 3. List of bacterial pathogens and their abundance levels in Thai vegetarians and non-vegetarians.

Bacterial pathogens	Non-vegetarians		Vegetarians		
	Relative abundance (%)	Prevalence (%)	Relative abundance (%)	Prevalence (%)	*p* Value
Escherichia coli	1.526	94.4	0.652	86.1	0.092
Escherichia hermannii	4.703[a]	97.2	0.716	86.1	0.032
Klebsiella pneumoniae	0.793	94.4	2.170[b]	91.7	0.032
Bilophila wadsworthia	0.166	86.1	0.014	30.6	<0.01

[a] Non-vegetarian group was significantly higher than the vegetarian group.

[b] Vegetarian group was significantly higher than the non-vegetarian group.

Discussion

The microbiotas of the two groups of Thai subjects consisting of 36 vegetarians and 36 non-vegetarians were analyzed using pyrosequencing. Based on both the % prevalence and relative abundance, the dominant gut bacteria of Thai vegetarians and non-vegetarians were *P. copri* and *Bacteroides* (especially *B. vulgatus*), respectively, which corresponded to the previous work done by Ruengsomwong *et al*. [45]. Using pyrosequencing analysis, additional dominant bacterial species were found. *Faecalibacterium prausnitzii* with high relative abundance of more than 10% showed up in both Thai subject groups. In fact, it was also the most abundant in a healthy adult's gut as reported by Miquel *et al*. [35]. *F. prausnitzii* exerts anti-inflammatory properties causing a lowering effect with Crohn's disease and inflammatory bowel disease [9, 16, 46]. These findings showed the possibly important role of *F. prausnitzii* against inflammation in the gut of all Thai subjects.

Both *Bacteroides* and *Prevotella* have already been reported as genera usually presented in the human gut regardless of nationality or continental geography [3]. *Bacteroides* was the most plentiful genus detected in the gut microbiota of those who consumed Western-style-food containing high protein and animal fat, whereas *Prevotella* was the most abundant genus in the gut microbiota of those who usually consumed a carbohydrate-enriched diet [8, 14, 50]. This result may be due to colonic fermentation, which can inhibit some gut microbiota. The carbohydrate fermentation results in an increased concentration of short-chain fatty acids, which in turn cause a decrease in pH from 6.5 to 5.5. *Bacteroides* species grow poorly at pH 5.5 [7]. This may be the reason why a low abundance of *Bacteroides* was found in vegetarians. Metabolite analysis should be further studied to complete this conclusion.

The most prolific bacteria found in the Thai subjects were genera *Faecalibacterium*, *Bacteroides*, and *Prevotella* and family Enterobacteriaceae. The present results are also consistent with the work done by De Filippo *et al*. [14] and Wu *et al*. [50]. De Filippo *et al*. [14] reported that *Prevotella* and *Xylanibacter* were major genera in the gut microbiota of African children from Burkina Faso, where the children were predominantly vegetarians. *Bacteroides* was the most abundant genus followed by *Faecalibacterium* detected in the gut microbiota of European non-vegetarian children living in urban Florence, Italy. Wu *et al*. [50] also studied the link between long-term dietary patterns and gut microbiota. His team found that the *Prevotella* enterotype was strongly associated with carbohydrate-enriched diets, whereas the *Bacteroides* enterotype was highly associated with protein and animal fat-based diet. Furthermore, *Prevotella* was previously observed in an agrarian society resident in USA [53]. However, from the fecal microbiota in vegetarian and omnivorous young women in southern India studied by Kabeerdoss *et al*. [23], it was reported that the microbial communities, especially the *Bacteroides-Prevotella* group, were similar in both the vegetarians and non-vegetarians, which are different from the current findings. This could be explained by the specificity of the primers for both *Bacteroides* and *Prevotella.* On the other hand, *Bacteroides-Prevotella* group, *Bacteroides thetaiotaomicron*, and *F. prausnitzii* as well as *Clostridium clostridioforme* were abundant in Slovania vegetarians [31]. It seemed that healthy Thai microbiotas were similar to other nationalities except Indian and Slovania, which had high abundance of both *Bacteroides* and *Prevotella* in vegetarians. However, high abundance of Enterobacteriaceae was also found in Thais but not in the others.

The core gut microbiota of Thai people comprised *F. prausnitzii, G. formicilis,* various species of *Bacteroides*, *P. copri*, short-chain fatty-acid-producing bacteria such as *Clostridium* sp., and some opportunistic pathogens belonging to the Enterobacteriaceae such as *Escherichia* sp. and *K. pneumoniae*. Within the core gut microbiota proposed, the genera *Bacteroides* and *Prevotella* were attributions for non-vegetarians and vegetarians, respectively, indicating a strong biomarker in Thais who had different food consumption styles. In addition, more than 1.5% of relative abundance of *Klebsiella* and *Escherichia* were found in the Thai subjects. This implied that Enterobacteriaceae may be a core flora in Thais. Nam *et al*. [39] reported that the core gut microbiota of Koreans contained *Bacteroides*, *Parabacteroides*, and *Provotella* in the Bacteroidetes phylum; and *Clostridium*, *Eubacterium*, *Faecalibacterium*, *Roseburia*, *Ruminococcus*, *Subdoligranulum*, butyrate-producing bacteria, and *Fusobacterium*; whereas for Americans, core gut flora were *Bacteroides putredinis* (recently designed as *Alistipes putredinis*), *Bacteroides vulgatus*, *Bifidobacterium longum*, *Blautia wexlerae*, *Coprococcus comes*, *Dorea formicigenerans*, *Eubacterium ramulus*, *Eubacterium rectale*, *Faecalibacterium prausnitzii*, and *Ruminococcus obeum* [33]. Therefore, the high prevalence of *Gemmiger* and Enterobacteriaceae (*Klebsiella* and *Escherichia*) included in Thai subjects distinguished their core gut microbiota from other nationalities.

Using next-generation pyrosequencing, Enterobacteriaceae, associated with bacterial disease, were found in both subject groups. The members of the Enterobacteriaceae found in fecal samples were bacterial species close to *E. coli*, *E. hermannii*, and *K. pneumoniae*, with more than 80% prevalence. There was a significantly higher % relative abundance of bacterial species close to *K. pneumoniae* found in the vegetarian group, as it is in fact part of the normal flora in fruit and vegetables such as banana [32], rice [1, 28] maize [7, 15, 41], potato [44], and lettuce [27]. In addition, it is also a part of the normal flora in the human GI tract [4]. Therefore, it is possible that living plant sources and suitable growth condition in the human GI tract would support its survival. Several published papers reported that *K. pneumoniae* can occasionally cause diarrhea [2, 19]. There was a high % relative abundance and prevalence of Enterobacteriaceae in non-vegetarians, specifically of *E. coli* and *E. hermannii*. *E. coli* is a normal component of the microflora in the human gut and is a versatile enteric pathogen. It is capable of causing diarrheal disease [25]. Although a rare strain of *E. coli* is an intestinal pathogen, in general, *E. coli* can produce vitamin K [5] and B12 [24]. *E. hermannii* is a human microflora found in stool specimens [6] and an opportunistic pathogen that can cause septicemia, meningitis, and neonatal brain infections in weakened and/or immunocompromised hosts [49]. Moreover, it can cause other diseases, including purulent conjunctivitis [43] and wound infection [42], especially when hands are not washed thoroughly after excretion and a wound or the eyes are touched. Therefore, it seemed that some species might be both opportunistic pathogens and useful microbes. The other member of the family Enterobacteriaceae, *Pantoea agglomerans*, is a plant pathogen but it can cause human infections such as infection of the bloodstream, joints/bones, and urinary tract [11, 21]. Even though those pathogens were found in the gut of some Thais, the people were all still healthy. This would imply that the bacterial balance of their core gut microbiota possibly supported their health.

Considering the high abundance and prevalence of these potential pathogens, the microbiota of both groups showed potential pathogen varieties of *Bilophila wadsworthia*, *E. coli*, *E. hermannii*, and *K. pneumonia*e. However, higher abundance levels of *Bi. wadsworthia*, *E. coli*, and *E. hermannii* were found in the non-vegetarian, whereas only *K. pneumoniae* was in vegetarians. The infection of some species were by external contamination such as food and feces as carriers, which are not a normal situation. These results implied that those pathogens were not always an infection risk. However, the different core gut microbiota of vegetarian and non-vegetarian groups can serve the occurrence of different potential pathogens to maintain their health.

The correlation of gut microbiota and personal characters showed significantly high and moderate positive correlations to the age and three species of *Bacteroides*, but had a negative association with *Roseburia faecis*. This study corresponded to the proposal of Ottman *et al*. [40] who reported that an increase in age caused a higher and lower abundance of Bacteroidetes and Firmicutes, respectively.

As it is known that diet, microbiota, and the occurrence of disease are linked, dietary modulation studies could provide valuable information to understand diet-microbiota-health issues [22]. Dietary modulation can be useful for medical application, as changing the host's microbiota can lead to better health. Wu *et al*. [51] suggested that the abundance of *Bacteroides* and *Prevotella* may be useful as a prognostic biomarker of disease. Therefore, the *Bacteroides* and *Prevotella* counts related to protein-based or carbohydrate-based diets of the Thai subjects, in combination with other bacterial species from the core gut microbiota, especially *F. prausnitzii*, could be used as a biomarker for predicting the health conditions of Thais and other Southeast Asians who have similar eating styles. *F. prausnitzii*, as a dominant species and a butyrate-producing bacterium, might be an interesting probiotic in the future. The overall balance in the composition of the gut microbial community and the presence of key species are important in ensuring homeostasis of the intestinal mucosa to enhance good health.

Acknowledgments

The authors would like to express their thanks to all the subjects who were willing to provide fecal samples for this research. This work was partially supported by the Center for Advanced Studies for Agriculture and Food, Institute for Advanced Studies, Kasetsart University, under the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, Ministry of Education, Thailand; a grant from the Asian Core Program, the Japan Society for the Promotion of Science (JSPS); and from The Corporation between the Thailand Institute of Scientific and Technological Research and the Academic Institute on Graduate Program Development.

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