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THESIS

GASTROINTESTINAL TRACT MICROBIOTA OF VEGETARIANS AND NON-VEGETARIANS AS HEALTH INDICATOR FOR THAI

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Preliminary study of microbiota in 7 Thai vegetarians and 6 non-vegetarians had been analyzed using polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE). Among the 186 DNA bands detected on polyacrylamide gel, 37 bands were identified mainly belonging to the group of *Bacteroides*, *Prevotella*, *Clostridium* and *Faecalibacterium prausnitzii.* A dendrogram of the PCR-DGGE divided the subjects into 2 clusters (vegetarians and non-vegetarians). Each cluster contained 2 sub-clusters: A1 and A2 for non-vegetarians cluster and B1 and B2 for vegeterians. However, there were still bacterial DNA bands left unidentified. Then after, pyrosequencing was used to reveal these unidentified bacterial DNA found in Thai vegetarians and non-vegetarians. In addition, the number of samples was also increased to 72 healthy Thai subjects. The pyrosequencing results revealed different tendencies for the microbiota in both sample groups. The non-vegetarians tended to have a higher abundance of *Bacteroides,* while the vegetarians had a higher abundance of *Prevotella*. It was also found that the non-vegetarians showed higher amount of Enterobacteriaceae, *Clostridium leptum*, and *F. prausnitzii-Subdoligranulum variabile* like bacteria groups. The amounts of other specific groups were not significantly different between the two sample groups. The difference of enterotypes between both sample groups may be due to their eating styles. *F. prausnitzii* and *Prevotella copri* can be used as the indicators for healthy Thai. Furthermore, the correlations between microbiota and various factors were trivially. The non-vegetarians have a chance to be overweight subjects due to their high BMI values. However, it would be better if Thai people would be aware to their hygienic foods, nutrition balance for good healthy and beneficial microbiota. However, all these results may cooperate with South East Asians microbiota with similar diet.

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Student's signature Thesis Advisor's signature

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Supatjaree Ruengsomwong

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LIST OF ABBREVIATIONS

° C	= degree Celsius
µl	= microliter
bp	= base pair
CFU/g	= colony forming unit per gram
DGGE	= denaturing gradient gel electrophoresis
DNA	= deoxyribonucleic acid
dNTP	= deoxyribonucleotide triphosphate
ect.	= et cetera (and other things)
g	= gram
h	= hour
M	= molar
mg	= milligram
min	= minute
ml	= milliliter
mM	= millimolar
ng	= nanogram
PCR	= polymerase chain reaction
rDNA	= ribosomal deoxyribonucleic acid
rpm	= revolutions per minute
sec	= second
temp	= temperature

GASTROINTESTINAL TRACT MICROBIOTA OF VEGETARIANS AND NON-VEGETARIANS AS HEALTH INDICATOR FOR THAI

INTRODUCTION

Human intestine is colonized by a large number of microorganisms, collectively termed microbiota. The development of intestinal microbiota in the human intestinal tract depends on microbes and their inoculum sizes at birth, consuming behavior and living environment (Salminen *et al*., 2006). Although some microorganisms are pathogens or harmless microorganisms, some microbes produce vitamins and other essential nutrients. They metabolize not only food that we cannot digest on our own but also drugs and toxins. Moreover, these beneficial microbes protect the host from infections and chronic inflammation as well as possibly many immune-based disorders (Phillips, 2009).

The commensal microbiota have specific functions for their host. These can be divided into three main groups. The first function is trophic function which includes control of proliferation and differentiation of epithelial cells and immune system maturation. The second function concerns the protective role of the microbiota by competitive exclusion mechanisms such as intestinal surfaces occupying and a systematic stability creating and antimicrobial compounds production. These mechanisms in turn prevent the invasion from exogenous microbes. The last one is the metabolic function including breakdown of non-digestible compounds such as resistant starch and plant polysaccharides by anaerobic fermentation. This fermentation generates shot chain fatty acids (SCFA) that serve as growth signals and fuel for the intestinal epithelium (Guarner and Malagelada 2003; Montalto *et al*., 2009).

Microbiota can be used as health indicators for diseases. For example, gastric cancer, *Helicobacter pylori* was detected in most gastric cancer patients but not in normal group by culturing, ELISA, immunoblotting, and immunohistochemistry tests (Dicksved *et al*., 2009). *H. pylori* was the causative agent of gastritis, peptic ulcers and the risk factor for the development of gastric cancer. It colonizes almost 40% of the population in many western countries. In 2008, *H. pylori* caused gastric cancer was 5.4% of all cancers caused

by infectious agent worldwide (Thun *et al*., 2010). Most individuals with gastritis will have an asymptomatic infection but about 10-20% of the infected individuals develop ulcer and 1-2% develop cancer (Sited by Kusters *et al*., 2006). In case of type II diabetes, the relative abundance of *Firmicutes* was significantly lower while the proportion of *Bacteroidetes* and *Proteobacteria* was higher in diabetes persons compared to their non-diabetes counterparts. The relationship between diabetes symptom and these bacteria was not clear but it was possible that Gram negative bacteria belonging to *Bacteroidetes* and *Proteobacteria* might lead to diabetes because the main compounds of outer membranes of Gram-negative bacteria were lipopolysaccharides. As the knowledge, lipopolysaccharides are potent stimulators of inflammation which can be exhibited endotoxemia. They might trigger an inflammatory response and played a role in the development of diabetes (Larsen *et al*., 2010). The other case is microbiota in allergic and non-allergic Japanese infants. The population of *Bacteroidaceae* was significantly higher in the allergic group at the first two month than in non-allergic group. No statistically significant difference was observed for the other bacterial populations. This trend might be a prospective marker for allergy crisis in infancy. The *Bacteroides fragilis* group induced significantly more helper T cell (T_H) type 2 cytokine (IL-6) but fewer TH I-type cytokines (IFN-y and IL-12) than those of *Bifidobacteria*. Also, it is known that *B. fragilis* LPS shown proinflammatory effects *via* the Toll-like receptor (TLR) 4 pathway, as enterobacterial LPS done, but that its potency was lower 100-1000-fold than *Enterobacteria*. On the contrary, it has been reported that *Bacteroides thetaiotaotnicron* attenuated inflammation by regulating intracellular signaling downstream of TLR signaling and NE-143 activation (Songjinda *et al*., 2007). Ley *et al*., (2006) found that the gut microbiota of obese individuals showed an elevated proportion of the phylum *Firmicutes* and reduced population of *Bacteroides* whereas their lean had a decreased *Firmicutes* / *Bacteriodetes* ratio.

Bacteria found in gastrointestinal (GI) tract play an important role of metabolism. *Bacteroides* species are some of the most common bacteria in the human gut. They involved in many important metabolic activities, including fermentation of carbohydrates, utilization of nitrogenous substances, and biotransformation of bile acids and other steroids. But *Bacteroides* can also cause many types of infections and abscesses in the GI tract elsewhere in the body. *Lactobacillus* is also the most commonly found in human gut. It produces lactase which can be used in probiotic products. For example, *L. acidophilus* supplements

are often given to lactose-intolerant individuals. *Clostridium difficile* is often acquired to antibiotic treatment patients in a hospital. Antibiotics alter the normal flora of the intestines, which allows for colonization by *C. difficile*. Once colonized, the bacteria release endotoxins that can cause colitis and severe diarrhea (Phillips 2009). *Butyrivibrio fibrisolvens* and *Roseburia inulivorans* found in human gut have linoleic isomerase activity. They can convert linoleic acid (*cis*-9, cis-12 C18:2), which can be found in dairy products, meat and fish, into stearic acid (C18:0) through conjugated linoleic acid (cis-9, trans-11 C18:2) and vaccenic acid (trans-11 C18:1), respectively. Stearic acid will be reduced by 2 carbon atoms per round in β-oxidation pathway. The electrons from β-oxidation will be passed through respiratory chain driving ATP synthesis (Devillard *et al*., 2007 and 2009). Moreover, some bacteria strains including *Lactobacillus acidophilus*, *L. casei*, *L. bulgaricus*, *L. plantarum*, *Bifidobacterium breve, B. infantis, B. longum, Streptococcus thermophiles,* and *Roseburia homimis* are able to convert linoleic acid into conjugated linoleic acid which is absorbed by the intestinal wall (Ewaschulk *et al*., 2006; Devillard *et al*., 2009; Alonso *et al*., 2003).

The gastrointestinal tract (GI tract) microbiota of human has been extensively studied but is yet not fully described. The techniques generally used for studying human microbiota can be broadly divided into cultivation and non-cultivation method. In the past, cultivation method is popular but it requires various kinds of culture medium. It took long time for culturing, colony purification and identification (Heginbothom *et al*., 1990). It is difficult to cover all of microorganisms in each environment because some microbes are fastidious. They need some factors to support their growth. The non-cultivation methods used in microbiota studies are molecular based techniques including 16S rRNA gene sequencing, T-RFLP, DGGE, qPCR and pyrosequencing identification (Tannock, 1999; O'Sullivan, 2000; Vaughan *et al*., 2000; Dethlesen *et al*., 2006). With new molecular based methods, they could be provide deeper knowledge on the microbiota in the GI tract of human. The understanding of GI tract microbiota is constantly growing and leads to involvement of the microbiota in human and other environments.

At present, many people are more attentive to their health even the medical advances has been developed. They focus on beneficial foods consumption especially fruits and vegetables which contain various vitamins, minerals and phytochemicals compounds. These gainful compounds have antioxidant activity, antimicrobial activity, stimulate the immune

system and associate with the development of cancer. For example, beta-carotene, lycopene, potassium, magnesium, vitamin E, folic acid, copper and fiber have reduced risk of coronary heart disease, cholesterol synthesis, and low density lipoprotein (LDL) receptor activity (Clinton 1998; Fuhrman *et al*., 1997; Dreher *et al*. 1996). In addition, garlic extracts have antimicrobial activity against *Staphyloococcus aureus* and *Enterococcus faecalis* (Ruddock *et al*., 2005). The early studies of health effects of vegetarians and vegan diets have shown low body mass index (BMI) and low concentration of plasma cholesterol (Key *et al*., 2006). These are the reasons that some people become vegetarians. Vegetarian products are popular in many countries such as Europe, India, Australia and Taiwan. They were labelled with the international label, so called V-label. The number of vegetarians in Italy is at 10% of population. It is the highest rate in the European Union. The number of vegetarians in Germany, Netherland, Switzerland and United Kingdom are 9%, 4.5%, 5% and 3% of population, respectively. In Americas, 9% of population of Brazil are vegetarians, 4% of Canadians are lacto-ovo-vegetarians whereas 2.5% of American are vegetarians and 0.5% are vegans. In Australia, vegetarian diets were promoted by animal rights organizations. Vegetarian week in Australia runs on 1-7 October every year. Five percentages of Australian are vegetarians. Some countries like China, Taiwan and Belgium have a national campaign of one day of vegetarian every week. It was proposed on national level too. Approximately 10% of Taiwanese population are vegetarians (Wikipedia 2012b, vegetarianism, retrieved on Feb 18, 2012). Almost 1% of Thai population are vegetarians. The number of vegetarian around the world tend to increase because of health concern. Number of Thai vegetarians is increasing as well.

Moreover, meat consuming may effect to our health. Consuming of meat or high intakes of saturated fats appear to be associated with an increased risk of colorectal cancer. Some microbes may play a role in the initial of colorectal cancer by converting dietary constituents to carcinogenic compounds. They are also able to transform bile acid or present in intestine into potential carcinogens such as nitrosamines, deoxycholic acid, lithocholic acid, ursodeoxycholic acid, fecapentaenes, ethionene and tryptophan metabolites (Wilson, 2005). Although cancer of colon and rectum are rare in developing countries including Thailand, high incidence rates were reported in Europe, North American, Australia and Japan. It should be noted that colon and rectum cancer incidence in Thailand is rising, probably due to accession of Western lifestyle (Khuhaprema and Srivatanakul, 2008).

Data from National Cancer Institute (NCI, Thailand) reported that colon and rectum cancer were the top three cancers found in male and female as shown in table 1 (Attasara and Buasom, 2007, 2008, 2009, 2010, 2011).

The piles of publication showed association between microbe and risk of cancer. Some microorganisms may support our health by decreasing risk factors of colon and rectum cancer. Some *Lactobacillus* and *Eubacterium aerogens* showed closet association with low risk of colon cancer. On the other hand, some *Bacteroides vulgatus, B. stercoris, Bifidobacterium longum* and *Bf. angulatum* were associated with increased risk of colon cancer because they were found in high amount in polyp patients and Japanese-Hawaiians who consumed a western-type diets, red meat diets (Moore and Moore, 1995). We probably enhance our health by changing in our consuming behavior and living environment.

Table 1 Statistic of cancer patients in percentage of number of patients

Year	Male				Female			
	Bronchus, lung	Liver and bile duct	Colon and rectum	Number of new cancer patients	Breast	Cervix uteri	Colon and rectum	Number of new cancer patients
2007	17.1%	14.8%	14.2%	n=1,219	40%	18.6%	6.2%	n=1,948
2008	16.2%,	10.5%	17.4%	n=1,114	43%	16.4%	8.8%	n=1,835
2009	18.5%	12.4%	14.1%	n=1,244	37%	14.4%	8.1%	n=2,070
2010	23.6%	17.3%	21.5%	n=1,189	47.8%	16.2%	10.4%	n=1,947
2011	15.5%	15.3%	16.2%	n=1,329	37.5%	14.4%	9.6%	n=2,012

Source: Attasara and Buasom (2007, 2008, 2009, 2010, 2011)

To the best of our knowledge, there has been no report on comparative study of microbiota between vegetarians and non-vegetarians in Thai people. As a consequence, we would like to investigate the composition of gut microbiota between Thai vegetarians and non-vegetarians in order to obtain the data which will be useful for human enhancement.

OBJECTIVES

1. To investigate the microorganisms in gastrointestinal tract from vegetarians and non-vegetarians (control group) in aspect of qualification and quantification using pyrosequencing and real-time PCR (qPCR) techniques, respectively.

2. To indicate the biomarker of healthy individual

LITERATURE REVIEW

The human GI tract is populated by complex communities of microorganisms. The microbiota of human GI tract represents a tremendous numbers of bacteria more than 10^{11} cells/g of content (Zoetendal *et al*., 2006). The dominant microorganisms differed with various factors including gut compartment, age and habit of consumption.

1. Microorganisms in gut compartment

The microorganisms are found thoroughly the gastrointestinal tract (Figure 1) from mouth to intestine, especially the colon. Each compartment is different in diversity and amount of microorganisms. The genus *Streptococcus* is numerically dominant in mouth. Most microflora in esophagus are similar to the species found in the oral cavity (Dethlesen *et al.,* 2006). Though, gastric acid kills many ingested microorganisms, the majority of miroflora in stomach are assigned to the Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes and Fusobacteria phyla. In addition, there are *Helicobacter pylori* and *Deinococcus*-related organism in human stomach (Bik *et al*., 2006). The bacterial diversity of community found in small intestine and colon increase from the jejunum to recto-sigmoid colon. The Gammaproteobacteria, including *Actinobacillus*, *Escherichia, Klebsiella, Enterobacter*, *Xenorhabdus, Proteus vulgaris* subgroup, are predominant bacteria in the feacal microbiota of elderly humans. These were also predominant in the jejunum ileum and caecum (Hayashi *et al*., 2005).

The numbers of microbial species found in GI tract are shown on Table 2. The species numbers are the highest in the mouth and reduce along its GI tract.

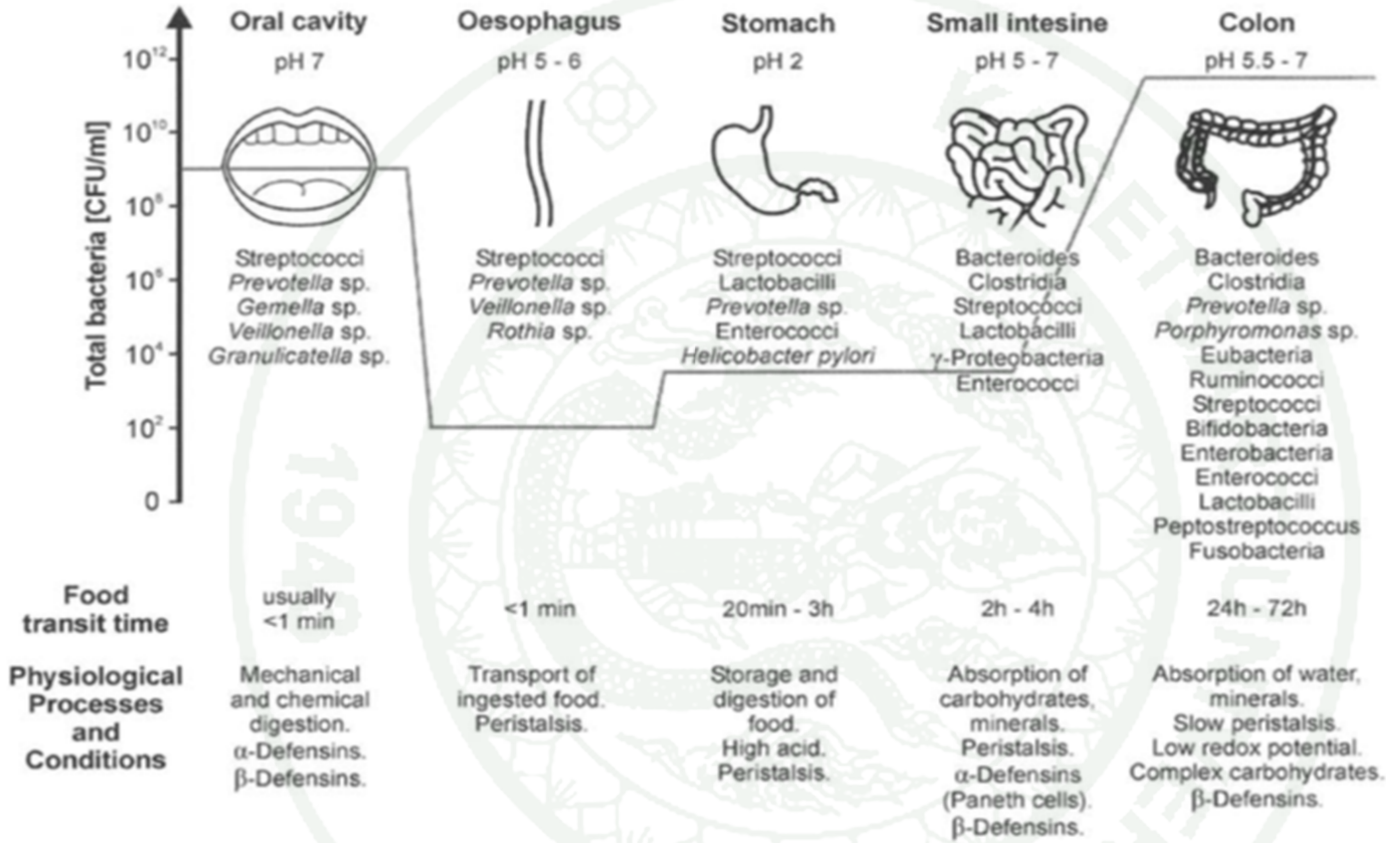

Figure 1 Physiologic conditions and bacteria genera generally found in human gastrointestinal tract compartment

Source: Huffnagle and Noverr (2008)

Table 2 Communities of the human microflora along the GI tract

Tissue	Number of microflora
Mouth	~700 species
Esophagus	95 species
Stomach	128 species
Small intestine	~10^4 to ~10^7 CFU/ml
Colon	~10^8 to ~10^{12} CFU/ml 300-400 species

Source: Dethlesen *et al.* (2006); Vaughan *et al.* (2000)

2. Intestinal microorganisms in relation to age

The microflora in infants are rapidly dominated by members of the genera *Bifidobacterium*, *Ruminococcus*, *Steptococcus, Enterococcus, Enterobacter, Clostridium, Lactobacillus*, *Escherichia coli* and uncultured bacterium. The representatives of intestinal microflora in adults are of the genera *Ruminococcus, Clostridium, Eubacterium, Bifidobacterium, Bacteriodes*, the family Coriobacteriaceae, *Streptococcus*, *Clostridium* cluster IV, IX, XIVa, and XVIII and uncultured bacterium. The number of bifidobacteria in the colon of adults is 10^{10}-10^{11} cfu/g, but this number decreases with age (De Vrese and Schrezenmeir, 2008). In elders, the major microflora belong to the *Bacteroides* group, *Escherichia* subgroup, *Enterobacter asburiae* subgroup, *Clostridium coccoides* group, *Clostridium leptum* subgroup, Lactobacilli, *Clostridium* cluster IV, IX, *Clostridium* subcluster XIVa, and Gammaproteobacteria. The detected microorganisms from various ages are given in Table 3.

Table 3 Distribution of microorganisms detected in different population in relation to age

Age	Microorganisms	Reference
Infant (0-12 months)	*Bifidobacterium breve*, *Bifidobacterium pseudocatenulatum*, *Ruminococcus* sp., *Steptococcus thermophilus*, *S. salivarius*, *Ennterococcus avium*, *E. raffinosus*, *Enterobacter aerogenes*, *E. asburiae*, *Clostridium neonatal*, uncultured bacterium	Favier *et al*., 2002
Infant (2 months)	*Escherichia coli,* *Enterococcus faecalis,* *Clostridium perfringens,* *Bifidobacterium longum, B. bifidum*	Blaut *et al*., 2002
Infant (0-6 days)	*Enterobacter* sp., *Enterbacter cloacae,* *Lactobacillus lactis,* *Leuconostoc citreum,* *Streptococcus mitis,* *Citrobacter,* *Clostribium difficle,* *Escherichia coli*	Park *et al*., 2005
Infant (0-2 months)	Family *Bacteridaceae* Family *Enterobacteriaceae* Genus *Bifidobacterium* Genus *Enterococcus* Genus *Lactobacillus* *Clostridium perfringens* group	Songjinda *et al*., 2005

Table 3 (Continued)

Age	Microorganisms	Reference
Adult (40 years old)	*Bacteroides ovatus,* *B. uniformis, Bacteroides* sp. *Eubacterium rectal,* *Ruminococcus obeum,* *Bifidobacterium* sp., *Clostridium* sp	Wilson and Blitchington, 1996
Adult (27-52 years old)	*Bacteroides* group, *Streptococcus* group, *Bifidobacterium* group, *Clostridium* cluster IV, IX, XIVa, XVIII and uncultured bacterium	Hayashi *et al*., 2002a
Adult (42-73 years old)	*Ruminococcus,* *Clostridium,* *Eubacterium,* *Bifidobacterium,* *Bacteriodes* and the family Coriobacteriaceae	Andersson *et al*., 2008
Elderly (65-90 years old)	*Bacteroides buccae-oris group,* *B. fragilis group, B. eggerthii, B. furcosus,* *B. oralis, B. putredinis, B. ureolyticus,* *Bacteroides* spp. *Fusobacterium mortiferum, F. varium,* *Fusobacterium* spp. *Selenomonas* spp. *Bifidobacterium adolescentis* , *B. longum,* *Bifidobacterium* spp.	Benno *et al*., 1989

Table 3 (Continued)

Age	Microorganisms	Reference
Elderly (65-90 years old)	*Eubacterium aerofaciens, E. contortum, E. lentum, E. rectal, Eubacterium* spp. *Lactobacillus gasseri, L. reuteri, L. salivarius Clostridium beijerinckii, C. butyricum, C. clostridioforme, C. cochlearium, C. innocuum, C. paraputrificum, C. ramosum, C. perffringens, Clostridium* spp.	Benno *et al*., 1989
Elderly (74-94 years old)	*Bacteroides* and relatives, *Clostridium* cluster IV, IX, *Clostridium* subcluster XIVa, and Gammaproteobacteria	Hayashi *et al*., 2003
Elderly (74-87 years old)	*the Bacteroides* group, *Escherichia* subgroup, *Enterobacter asburiae* subgroup, *Clostridium coccoides* group (e.g. *Ruminococcus hansenii* subgroup, *R. gnavus* subgroup, *Butyrivibrio fibrisovens* subgroup), *Clostridium leptum* subgroup, *Lactobacilli* (e.g. *Lactobacillus reuteri* subgroup, *L. mali* subgroup, *Streptococcus salivarius* subgroup, *and S. pneumonia* subgroup)	Hayashi *et al*., 2005

3. Intestinal microorganisms in habit of consumption in aspect of vegetarian diets

The intestinal microflora in a strictly female Japanese vegetarian who has been taken only vegetarian soup (200 ml/day) for 13 years represented *Clostridium* rRNA cluster IV, XIVa, XV, XVI, and XIII belonging to the Firmicutes phylum as the dominants. *Cytophaga-Flexibacter-Bacteroides* were followed the Firmicutes. *Bifidobacterium* and Proteobacteria were rare in vegetarian. This investigation was using three methods, culturing

on medium 10 under anaerobe condition, cloning and terminal fragment length polymorphism (T-RFLP) (Hayashi *et al*., 2002b).

Liszt *et al*. (2008) reported that *Clostridium* rRNA cluster IV were lower in vegetarians than omnivores. *Bacteroides* were abundant in vegetarians. Moreover, vegetarians showed 12% higher count of bacterial DNA. These differences were not significant due to high interindividual deviation. The mean proportion of *Bifidobacterium* in vegetarians and omnivores were similar.

The other report focusing on microbiota in vegetarians reported that total counts of *Bacteroides* spp., *Bifidobacterium* spp., *Escherichia coli* and *Enterobacteriaceae* spp. were significantly lower in vegan samples than in controls, whereas others (*E. coli biovars*, *Klebsiella* sp., *Enterobacter* sp., other *Enterobacteriaceae*, *Enterococcus* sp., *Lactobacillus* sp., *Citrobacter* sp. and *Clostridium* sp.) were not. Subjects on a vegetarian diet ranked between vegans and controls. The total microbial count did not differ between the groups. In addition, subjects on a vegan or vegetarian diet showed significantly lower stool pH than did controls, and stool pH and counts of *E. coli* and *Enterobacteriaceae* were significantly correlated across all subgroups (Zimmer *et al*., 2012).

4. The vegetarianism effects to health

Generally, the definition of vegetarian is a person who does not eat meat, fish, poultry, or, in some cases, any food derived from animals such as eggs or cheese, but subsists on vegetable, fruits, nuts, grain, etc. The vegetarianism can be classified in 4 types. Firstly, ovo-vegetarian is a person who eats plant food and eggs but not dairy products. Secondly, lacto-vegetarian is a person who eats plant food and dairy products but not eggs. Thirdly, ovo-lacto-vegetarinan is a person who eats both eggs and dairy products and plant product. Lastly, vegan is a person who does not eat all animal products, including eggs, dairy and all other animal-derived ingredients. These vegetarian diets are rich in carbohydrates, dietary fibre, carotenoids, folic acid, vitamin C and E and magnesium (Mg), but low in protein, saturated fat, retinol, vitamin B12 and zinc (Zn).

The advantages of vegetarian diets were reported. The previous study reported that Western vegetarians had lower BMI than non-vegetarians about 1 kg/m^2. Total plasma cholesterol in vegetarians was lower than non-vegetarians about 0.39 mmol/l in men and

0.35 mmol/l in women reported by European Prospective Investigation of Cancer and Nutrition (EPIC-Oxford) (Key *et al*., 1999) and 0.61 mmol/l in Health food Shoppers Study (Key et al., 2006). Vegans had the lowest BMI values of all vegetarians (Craig 2010). In addition, vegetarians had a lower blood pressure than non-vegetarians, with differences of 2-10 mmHg in systolic or diastolic pressure (Beilin *et al*., 1988). Blood lipids and BMI were significantly lower in African American vegans than in lacto-ovo-vegetarians (Toohey *et al*., 1998). In case of Asian vegetarians, Taiwanese vegetarians had lower total cholesterol, LDL-cholesterol and high homocysteine levels than non-vegetarians, but they had a higher cardiovascular risk than non-vegetarians (Chen *et al*., 2007).

Key *et al*. (2009) reported that the incidence of stomach, pancreas, lung, breast, ovarian, prostate cancer and malignant neoplasm combined was lower among vegetarians than among meat eaters, but colorectal cancer was higher in vegetarians than meat eaters. In contrast, World Cancer Research Fund, American Institute for Cancer Research concluded that high intake of red and processed meat causing colorectal cancer was convinced (sited by Key *et al*., 2009). This conclusion was confirmed by Chan *et al*., (2011) who reported that high intake of red and processed meat was associated with significant increased risk of colorectal, colon and rectal cancers. Furthermore, both red and processed meat intakes were positively associated with cancers of the colorectum and lung. Moreover, red meat intake was associated with an elevated risk for cancers of the esophagus and liver (Cross *et al*., 2007).

However, vegetarians and vegans may have particularly lack of vitamin B12, D and calcium (Ca). Although dairy and eggs are good sources of vitamin B12 for lacto-ovo-vegetarian, vegans should regularly consume vitamin B12–fortified foods, such as fortified soy and rice beverages, certain breakfast cereals and B12–fortified nutritional yeast, or take a daily vitamin B12 supplement. Fermented soy products, leafy vegetables, and seaweed cannot be considered a reliable source of active vitamin B12. No unfortified plant food contains any significant amount of active vitamin B-12. Adequate vitamin B12 intake is especially important during pregnancy and lactation. Vitamin D plays an important role in immune function, the reduction of inflammation, and reducing the risk of chronic diseases. Vitamin D insufficiency has been linked to a wide variety of diseases including type I diabetes, multiple sclerosis, rheumatoid arthritis, colorectal cancer, heart disease, and infectious diseases (Craig, 2010). To ensure an adequate vitamin D status, vegans must regularly consume vitamin D–fortified foods such as soy milk, rice milk, orange juice,

breakfast cereals, and margarines that are fortified with vitamin D. A daily supplement of 5–10 μg of vitamin D would be necessary. The supplement would be highly desirable for elderly vegans. To ensure adequate calcium in the diet, calcium-fortified plant foods should be regularly consumed the traditional calcium sources for a vegan such as green leafy vegetables, tofu. The calcium-fortified foods include ready to eat cereals, calcium-fortified soy and rice beverages, calcium-fortified orange and apple juices, and other beverages. The bioavailability of the calcium carbonate in the soy beverages and the calcium citrate malate in apple or orange juice is similar to that of the calcium in milk. Tricalcium phosphate–fortified soy milk was shown to have a slightly lower calcium bioavailability than the calcium in cow milk (Craig 2009).

5. Methods for analysis of the intestinal microflora

Each of the techniques used for the assessment of the diversity of the human gastrointestinal microbiota has its limitations and induces biases in the obtained portray of microbial diversity.

5.1 Culture techniques and characterization for intestinal microflora

Before molecular techniques era, understanding of the diversity and role of individual microflora has been successful by culture techniques with selective culture and non-selective culture methods. The samples are homogenized in a sterile liquid such as peptone water buffer, normal saline before culturing in selective or/and non-selective media.

Non-selective media are generally used to estimate total numbers of both aerobes and anaerobes in human gastrointestinal tract. These non-selective media are rumen fluid-glucose-cellobiose agar (RGCA), modified medium 10, plate count agar, brucella blood agar (BBA) supplemented with 0.5% sheep blood, 1 mg/ml vitamin K and 5 mg/ml hemin, and brain heart infusion (BHI) (O'Sullivan, 2000). Some bacteria cannot grow on these media because they require extra supplements. So, selective culture methods are used in parallel.

There are researches using non-selective media for analysis of gut microflora. For example, the normal flora of fecal samples of 20 Japanese-Hawaiians are isolated from RGCA. There are various bacterial group such as *Bifidobacterium adolescentis, B. longum,*

B. infantis, B. breve, Eubacterium aerofaciens, E. eligans, E. biforme, E. rectal, *Lactobacillus acidophilus, L. leichmannii, L. salivarius, L. fermentum, L. minutes*, *Bacteroides flagilis, B. hypermegans, B. ruminicola, Fusobacterium prausnitzii, Ruminococcus bromii, R. callidus, Clostridium ramosum, Streptococcus salivarius* (Moore and Holdeman, 1974). Benno *et al.* (1989) have used 4 non-selective agar plates, modified medium 10, Eggerth- Gagnon (EG), glucose-liver-blood (BL), and trypticase soy with 5% horse blood, to determine those fastidious anaerobes and anaerobes. Moreover they have used selective agar plates such as neomycin-brilliant green taurocholate-blood (NBGT) for *Bacteroides* spp., neomycin-Nagler (NN) for lecithinase-positive clostridia, triphenyltetrazolium choride-acridine orange-thallous sulfate-esculin-crystal violet (TATAC) for streptococci, phenylalcohol-egg yolk suspension (PEES) for staphylococci, potato dextrose (PD) for yeasts and molds, deoxy-cholate-hydrogen sulfide-lactose (DHL) for enterobacteria, and nalixidic acid-cetrimide (NAC) for *Pseudomonas*.

In general, specific bacterial genera are achieved by plating on selective media for selective method. Several selective agents such as bile, esculin or antibiotics can be used for selective enrichment for *Bacteroides* species. Common selective media used for *Bifidobacterium* for example YN-6, Pentuey's selective media (PSM), BS1, BIM-25 and Beerens medium, have been used for analysis of the human intestine microorganisms. *Bifidobacteria* selective agents in these media mainly include antibiotics such as kanamycin, naladixic acid, paramycin and polymyxin B. Sometimes, propionic acid was used as a selective agent. The Lactobacilli are commonly cultured from intestinal or fecal samples using Rogosa (Difco) or acidified Man Rogosa Sharpe (MRS; Difco) or LAMVAB media. The Clostridia can be isolated by cultivating on novobiocin colistin agar (NCA) and colistin crystal violet agar (CCA). Enterococci and Streptococci can be isolated using Stanetz-Bartley (SB) medium, also called Bacto m Enterococcus Agar (Difco), or oxolinic acid-esculin-azide (OAA). In addition, Enterobacteriaceae can be isolated using MacConkey agar (Difco). However, they all have the inherent disadvantages of not absolute selectivity and toxicity against certain strains within genus (O'Sullivan 2000).

Furthermore, Matsuki *et al.* (2004) investigated human intestinal Bifidobacteria by plating on TOS agar. This medium consists of 10g trypticase, 1g yeast extract, 3g KH_2PO_4, 4.8g K_2HPO_4, 3g $(NH_4)_2SO_4$, 0.2g $MgSO_4$, 0.5g L-cysteine, 10g transgalactosylated oligosaccharides, and 15g agar per one liter of medium. The results were

similar to both Fluorescent *in situ* Hybridization (FISH) and Quantitative Real-time Polymerase Chain Reaction (qPCR).

It is necessary to confirm the genus identity and also further characterization of isolated colonies to the species level is needed. This characterization requires morphological and biochemical tests which can be obtained from the Bergy's Manual of Systematic Bacteriology. The confidence level of the species identification will increase with more tests that are carried out.

Some specific enzymes and metabolites in fecal samples can indirectly give information on the presence of specific microflora. It can allow the analysis of a large number of individuals quite rapid. Measurement of increased short chain fatty acids (SCFA) in faeces can point to increase in metabolic activities of lactic acid bacteria. For example, *Lactobacillus casei* GG in children significantly increased the total SCFA concentration and *Bifidobacteria* increased acetate production. Azoreductase and nitroreductase can generate toxic metabolites in the intestine, while species of *Bacteroides, Eubacterium* and *Clostridium* are likely candidates responsible for these two enzymes. More studies are needed to accurately correlate specific fecal enzymes with specific group of bacteria. Moreover, direct microscopic analysis does give a good indication of the total microbial population numbers present in faeces (O'Sullivan, 2000).

5.2 Molecular techniques and characterization for intestinal microflora

The culture techniques for isolation of microflora from the human intestine are lack of accuracy. It is laborious and time consuming because some species have slow growth and some of them cannot grow in common medium. Selective media are not available for most of the strict anaerobes (Vaughan *et al.*, 2000). At present, molecular techniques widely used to investigate the human intestinal microbiota are 16S rDNA cloning, Fluorescent *in situ* Hybridization (FISH), Denaturing Gradient Gel Electrophoresis (DGGE), Temperature Gradient Gel Electrophoresis (TGGE), Quantitative Real-time Polymerase Chain Reaction (qPCR), Terminal Restriction Fragment Length Polymorphism (T-RFLP), and pyrosequencing.

5.2.1 16S rDNA cloning

The 16S ribosomal RNA (16S rDNA) contains conserved and variable regions across all bacteria species. The variable region sequences are sometimes highly species-specific. So, 16S rDNA sequence similarities can be used to identify bacterial species in the analysis of bacterial communities. Classification and evaluation of microbial evolutionary relatedness was first developed by Woese and coworkers in 1987 (O'Sullivan, 2000). The amplification of 16S rDNA sequence from bacteria can be done by using universal primers and species-specific primers. Then the amplified 16S rDNA sequences are cloned into cloning vectors. The 16S rDNA clones are screened and sequenced. Some sequences may have been cloned more than once. The sequences can be compared to the rDNA database.

The suspended fecal samples are plated on medium 10 and made 16S rDNA clone libraries proposed by Wilson and Blitchington (1996) and Hayashi's team (2002a). The extracted DNA is amplified by universal primer. Wilson and Blitchington used primer PC5B and P3mod and TA cloning kit from Invitrogen (USA). They reported that the microflora in human colon are *Bacteroides* sp., *B. ovatus, B. uniformis*, *Eubacterium rectal*, *Clostridium* group, *Bifidobacterium, Betaproteobacterium, Ruminococcus lactaris*, and *R. obeum* (Wilson and Blitchington, 1996).

This technique has greatly increased our understanding of the phylogenetic relationships between the microflora in the human intestine (O'Sullivan, 2000; Vaughan *et al*., 2000) because the variable region sequences are sometimes highly species-specific (Tannock, 1999). In contrast, this technique has some limitations. These limitations are laborious for large scale cloning (Vaughan *et al*., 2000), incomplete nucleotides sequence of 16s rRNA genes from each microbial variety analyzed according to their similarity base, and high similarity of different species. Many species, especially among *Enterobacteriaceae*, have more than 98% 16s rRNA gene sequence similarity with other related species (Rajilić-Stojanović *et al*., 2007).

Table 4 shows cloning vectors and universal primer for human microbiota analysis widely used.

Table 4 Universal primers and cloning vectors used for human microbiota analysis

Primer	Sequence (5′→3′)	Product Size (bp)	Cloning vector / competent cells	Reference
8f	CACGGATCCAGAGGTTGAT (C/T)(A/C)TGGCTCAG	1,500	pGEM-T vector (Promege, USA)/ *E. coli* JM109	Zoetendal *et al*., 1998
1510r	GTGAAGCTTACGG(C/T)TAC CTTGTTACGACTT			
27F	AGAGTTTGATCCTGGCTCA G	1,466	pCR®2.1/ One Shot®INαF′ (Invitrogen, USA)	Hayashi *et al*., 2002b
1492R	GGTTACCTTGTTACGACTT			
27F	AGAGTTTGATCMTGGCTCA G	316	pGEM T-easy vector (Promege, USA)/ *E. coli* DH5α	Park *et al*., 2005
342R	CTGCTGCSYCCCGTAG			
27F	AGAGTTTGATCMTGGCTCA G	1,465		
1492R	TACGGYTACCTTGTTACGA CTT			
63F	CAGGCCTAACACATGCAAG TC	1,325		
1387R	GGGCGGWGTGTACAAGGC			
BAC-338f	ACTCCTACGGGAGGCAG	468	pGEM T-easy vector (Promege, USA)	Zweilehner *et al*., 2009
BAC-805R	GACTACCAGCGTATCTAAT CC			

Sequence in IUPAC code; M = A/C, S = G/C, W = A/T, Y= T/C

5.2.2 Fluorescent *in situ* Hybridization (FISH)

Fluorescent *in situ* Hybridization (FISH) is a technique to hybridize fluorescent labeled oligonucleotide probes directly to cells fixed on a glass slide. The fixing process permeates the cells to allow accession of the shot probes to nucleic acid inside the target cell. This hybridization can be carried out on glass slides. The cells with the hybridized fluorescent probe can be subsequently visualized by fluorescent microscopy. Probes are 15-20 nucleotides in length. These probes are labeled covalently at the 5'-end

with a fluorescent dye. Two recent improvements of the basic FISH are the multiplex FISH and the multicolor FISH. The multiplex FISH essentially consists of independent multiple hybridization by several probes carrying different fluorescence tags. The multicolor FISH uses species-specific probes. These probes are labeled with more than one fluorochrome in different ways, singly or combination. FISH has indicated the number of bacteria in human faecal samples is approximately ten-fold higher than estimated using standard culture techniques. This technique can reveal what specific genes are expressed by microbiota *in situ* in human intestine (O'Sullivan, 2000; Cocolin and Ercolini, 2008).

Langendijk *et al*. (1995) developed 16S rRNA hybridization probes for *Bifidobacterium* species of the human fecal flora. It is whole-cell hybridization with fluorescien isothiocyanate-labelled probes and monitored by videomicroscopy. They compared the amount of *Bifidobacterium* with cultural method. It is concluded that all bifidobacteria in faeces are culturable. The total of bifidobacteria is overestimated by almost 10-fold when cultural method is used.

Microbiota of two hundred and thirty healthy subjects from four European locations in France, Germany, Italy and Sweden was performed by a cross-sectional study on intestinal microbiota composition. The participants of this study were designed to two age groups: 85 volunteers of 20-50 years (mean age, 35 years) and 145 volunteers of higher 60 years (mean age, 75 years). A set of 14 group- and species-specific 16S rRNA-targeted oligonucleotide probes was applied to the analysis of fecal samples by FISH coupled with flow cytometry. The interactions of country-age were observed for the German and Italian study groups. These interactions were inverted for the predominant bacterial groups *Eubacterium rectal-Clostridium coccoides* and *Bacteroides-Prevotella*. Differences between European populations were observed for *Bifidobacterium* group only. Bifidobacteria proportions were two to three folds higher in Italian population than any other group and this effect was independent of age. Higher populations of enterobacteria were found in all elderly volunteers independent of the location. Gender effects were observed for the *Bacteroides-Prevotella* group. They were higher levels in males than in females. In conclusion, age-related differences in microbiota were detected but not differed in between the study populations from the four countries (Mueller *et al*., 2006).

FISH is powerful, provides high discrimination level, reproducibility and easy interpretation. The drawbacks of this technique are moderate to high cost, long

time consuming (for culture), probe design requirement. Molecular genetics of gene probe and target sequence must be known (Vanughan *et al*., 2000; Cocolin and Ercolini, 2008).

5.2.3 Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing Gradient Gel Electrophoresis (DGGE) is a family of electrophoresis method for separation of nucleic acids, DNA or RNA. It was invented by Leonard Lerman and Stuart Fisher. This fingerprinting technique works by applying a small sample of nucleic acids (PCR product) to polyacrylamide gel that contains formamide and urea as denaturing agents. PCR products are amplified from microflora using high GC content primer or GC clamp. During this process, PCR products encounter increasingly higher concentration of denaturing agents as they migrate through an electrophoresis gel. When double-stranded DNAs reach to melting point at threshold denaturant concentration, the weaker melting domains of double-stranded PCR product will begin to denature. They dissociate partially to single strand and stop migration in polyacrylamide gel. Different sequences of DNA from different of microorganisms will denature at different denaturant concentration. The results display in a pattern of bands like fingerprint. Each band represents a different bacterial population present in the community. This technique is used for the separation of DNA segments of approximately 200-400 bp (Tannock, 1999; O'Sullivan, 2000; Rajilić-Stojanović *et al*., 2007).

Satokari *et al*. (2001) developed a method for quantitative analysis of complex bifidobacterial communities based on PCR using *Bifidobacterium* genus-specific primers and DGGE. PCR product from different bifidobacterial species showed a good separation upon DGGE. In this study, *Bifidobacterium adolescentis* was found to be the most common species in faeces of the human adult subjects.

A Lactobacillus group-specific PCR primer is developed to selectively amplify 16S rDNA from lactobacilli and related lactic acid bacteria, including members of the genera *Leuconostoc*, *Pediococcus* and *Weissella*. The primer is demonstrated by using *Lactobacillus*-specific PCR and DGGE of 16S rDNA amplicons. The *Lactobacillus* community in 3 adults over a 2-year period showed variation and stability depending on the individual. The *Lactobacillus* community is observed during the first 5 months of an infant's life. In conclusion, the combination of specific PCR and DGGE analysis of 16S rDNA amplicons allows the diversity of group of bacteria that are present in low members in

specific ecosystems to be characterized, such as lactobacilli in the human GI tract (Heilig *et al*., 2002).

Fecal samples from two healthy babies are analyzed from birth for more than 10 months of life by using PCR-DGGE and 16S rDNA sequencing. DGGE profiles of the dominant populations in the intestines of infants showed the bacterial diversity. The profiles of the first few days of life are simple. But they become more complex as the bacterial diversity increased with age in both babies. The bacteria included members of the genera *Bifidobacterium, Ruminococcus, Enterococcus, Clostridium,* and *Enterobacter.* The genera *Bifidobacterium* and *Ruminococcus* are dominated in intestinal microflora. They are estimated by the intensities of the bands. More than half of cloned rDNA sequences are exhibited less than 97% identity with sequences of known bacteria in databases. It is shown that using PCR-DGGE and 16S rDNA sequence analysis together resulted in a description of bacterial colonization in intestinal ecosystem of infant. So, it allowed visualization of bacteria that are difficult to cultivate or to detect by other methods (Favier *et al*., 2002).

The bacterial community in the Japanese neonatal gastrointestinal tract was determined by monitoring 16S rDNA diversity in fecal samples using PCR and DGGE. This study showed a certain pattern infants without antibiotic treatment. Aerobic bacteria, *Pseudomonas,* appeared first. Then facultative anaerobes, *Enterococcus*, *Streptococcus* and *Enterobacteriaceae* were immediately replaced through the first month. Finally, strictly anaerobe, *Bifidobacterium*, was appeared (Songjinda *et al*., 2005).

Fecal samples were collected from 21 infants at 1 and 4 months of life to examine the relationship between the bacterial diversity of the gut and the development of eczema in early life by DGGE. From 21 infants, 9 infants were diagnosed with eczema by the age of 6 months as in cases and the others were as controls. The number of electrophoretic bands and the Shannon index of diversity (H') were used as indicators for comparison of the microbial diversity of cases and controls. From the results, fecal microbial diversity of control subjects had significantly greater than children with eczema at age 1 and 4 months. The microbial diversity from 1 to 4 months of age is significantly increased in controls but not in children who developed eczema. It is suggested that microbial diversity is associated with the development of eczema in early life (Forno *et al*., 2008).

DGGE is routinely used in many microbiological laboratories worldwide as a molecular tool to compare the diversity of microbial communities and to monitor population dynamics. It is reliable, reproducible and inexpensive. An additional strong feature of this technique is possibility of identifying community members by sequencing of excised bands or by hybridization analysis with specific probes, which is not possible with other fingerprinting techniques (Muyzer, 1999). This technique is rapidly comparative analysis and good reproducibility. It provides detection of specific groups and semi-quantitative. However, it requires a lot of standard strains for reference (O'Sullivan, 2000 and Vaughan *et al*., 2000). The main limitation is that the fingerprints do not generate directly translate into taxonomic information. It is necessary to excise and re-amplify DNA fragments to 16S rDNA gene sequences (Cocolin and Ercolini, 2008).

5.2.4 Temperature Gradient Gel Electrophoresis (TGGE)

Temperature Gradient Gel Electrophoresis (TGGE) is a family of electrophoresis method for separation of nucleic acids like DGGE. It relies on temperature dependent changes in structure of double stranded DNA. This method consists of the amplification of the 16S rDNA gene from different bacterial populations, followed by the separation of DNA fragment. The process starts with a double stranded DNA molecule of a few hundred basepairs in length. At room temperature, in the presence of at least mM of salt, the double stranded form is quite stable. DNA molecule contains a negatively charged molecule (anion). DNA molecule will move to the positive electrode when it is in the presence of an electric field. A gel is a molecular mesh, with holes roughly the same size as the diameter of the DNA string. The DNA will attempt to move through the mesh, and for a given set of conditions. The speed of movement is roughly proportional to the length of the DNA molecule. When the temperature is raised as a linear temperature gradient, the two strands of the DNA start to come apart or melting. At some high temperature, the two strands will completely separate. However, at some intermediate temperature, the two strands will be partly separated with some parts of the molecule are still double stranded and part single stranded. DNA molecules with different sequences may have different melting behavior. They will stop migration at different positions along the gel. TGGE generated patterns could provide a preliminary ecological view of predominant species, increasing or decreasing in complex microbial communities. This is observing appearance or disappearance of specific amplicons in the denaturing gel (Sailey, 2012; Muyzer, 1999 and Cocolin and Ercolini, 2008).

Zoetendal *et al*. (1998) investigated the diversity of the predominant bacteria in the human gastrointestinal tract by using TGGE profile of 16S rRNA and rDNA analysis. TGGE profile of fecal PCR amplicons from 16 individuals showed different profiles, with some bands in common. These profiles derived from 16S rRNA and rDNA amplicons showed similar patterns. However, the intensities of bands with similar mobilities differed in some cases, indicating a different contribution to the total active fraction of the prominent fecal bacteria. PCR amplicons of one subject were identified by cloning and sequence analysis. Forty five of seventy eight clones matched 15 bands but 33 clones did not match any visible band in the TGGE pattern. The sequences matching 15 bands showed 91.5-98.7% homology to sequences derived from different *Clostridium* clusters.

To analyze the biodiversity of active bacteria in the patients with ulcerative colitis (UC) and compared with that of health subjects, faeces were collected from 9 patients with UC and from 9 health controls. Since previous studies of the endogenous microbiota in patients with UC have not taken bacterial activity into account. The bacteria with high transcriptional activity might have a more important pathophysiological role. Total DNA and RNA were compared by means of TGGE. The interesting bands were excised, sequenced and identified by comparison with the GenBank database (NCBI). The dominant species diversity based on RNA-derived TGGE profiles was significantly for UC patients lower than health controls. The band was sequences for 6 patients and corresponded to *Escherichia* coli. The biodiversity of active bacteria in the dominant fecal microbiota of patients with UC is lower than healthy subjects. ***E. coli*** is more represented in the active microbiota of UC patients (Sokol *et al*., 2006).

TGGE is a well molecular tool in environmental microbiology that allows the study of complexity and behavior of microbial communities. The technique is reliable, reproducible and inexpensive. An additional strong feature of this technique is possibility of identifying community members by sequencing of excised bands or by hybridization analysis with specific probes, which is not possible with other fingerprinting techniques (Muyzer, 1999). The main limitation of TGGE is that the fingerprints do not generate directly translate into taxonomic information as samel as DGGE (Cocolin and Ercolini, 2008).

5.2.5 Quantitative Real-time Polymerase Chain Reaction (qPCR)

The real-time PCR was improved by fluorescence which could bind to double stranded DNA. The kinetics of fluorescence accumulation during thermocycling was directly related to the starting number of DNA copies. This is a starting point of real-time qPCR. In classical PCR, the product can be run on a gel for detection of this specific product after the end of amplification. In real-time PCR, this step can be avoided since the technology combines the DNA amplification with immediate detection of product in a single tube. The number of cycles to reach a certain detection level is an indication of abundance of the original template. It can be estimated from a standard curve that created by using a serial dilution series of standard DNA. It is also less time consuming than gel based analysis and can report a quantitative result. Typical qPCR approaches originally developed with the 5' fluorogenic exonuclease (Taqman) assay representing the latest and widest applied development. It uses a labeled with fluorescent dyes internal probe as standard PCR amplification primers. Taqman chemistry provides in tube. Real-time detection of PCR product accumulation occurs during each amplification cycle and at very early stages in amplification process. Using DNA as starting material, knowledge of the absolute composition, abundance of microbial community, dynamics of individual populations, organisms or genes within that community can be obtained. Using RNA as a template and a real-time reverse transcription PCR assay, highly sensitive quantification of mRNA to measure levels of the gene expression within a microbial population is possible. The qPCR technique is currently very popular to introduce in quantifying bacteria in human and animal fecal samples (Songjinda et al., 2007 and Cocolin and Ercolini, 2008).

Quantitative PCR detection method has been developed and applied to the analysis of bifidobacteria distribution in human intestine by combining real-time PCR with *Bifidobacterium* genus- and species-specific primers. It was found that the method could be applied to the detection of *Bifidobacterium* in faeces when it was present at concentration of $<10^6$ cells per g of faeces. Because real-time PCR detection of serially diluted DNA extracted from cultured bifidobacteria was linear for cell counts ranging from 10^6 to 10 cells per PCR assay. The *Bifidobacterium adolescentis* group, the *Bifidobacterium catenulatum* group and *Bifidobacterium longum* were three predominant species from the faeces of 46 healthy adults (age 25-59 years). Six of forty six healthy adults were examined in the population changes and for composition of *Bifidobacterium* species in human

intestinal flora over an 8-month period. The result showed that the composition of bifidobacterial flora was stable throughout the test period (Matsuki *et al*., 2004).

Primer sets of six predominant bacterial groups in the intestinal infants were used for real-time PCR to estimate quantities of each population in the faeces. These primer sets targeted for *Bacteroidaceae*, *Enterobacteriaceae,* bifidobacteria, enterococci, lactobacilli and the *Clostridium perfingens* group. The bacterial compositions of faeces were monitored in the first 2 months for 15 infants born in Japan, including 8 subjects who developed allergy by the age of 2 years. The population of *Bacteroidaceae* was significantly higher in the allergic group at the ages of 1 month and 2 months than in the non-allergic group. No statically significant difference was observed for the other bacterial populations (Songjinda *et al*., 2007).

The human fecal microbiota from three age-groups including infants, adults and elderly was assessed by qPCR. The counts of major bacterial groups: *Clostridium leptum, Clostridium coccoides, Bacteroidetes, Bifidobacterium, Lactobacillus* and *Escherichia coli* were estimated. The microbiota of infants was generally characterized by low levels of total bacteria. *C. leptum* and *C. coccoides* highly represented in infants. Elderly subjects exhibited high level of *E. coli* and *Bacteroidetes.* The ratio of *Firmicutes* to *Bacteriodetes* evolves during different life stages, for infants, adults and elderly subjects are 0.4, 10.9 and 0.6, respectively (Mariat *et al*., 2009).

This technique is good reproducibility, easy interpretation and quantitative population dynamics of microbial group. The disadvantages of qPCR are high cost of chemical reaction and overestimate when primer dimer or non-specific sequence is occur (Cocolin and Ercolini, 2008).

The species-specific primers on Table 5 were used for quantitative analysis of microbial intestinal human.

Table 5 Species-specific primers used for human microbiota analysis

Primer	Target	Sequence (5′→3′)	Reference
Fwd primer	*Bifidobacterium*	GCGTGCTTAACACATGCAAGTC	Zweilehner
Rev primer		CACCCGTTTCCAGGAGCTATT	*et al*., 2009
AllBac296f	*Bacteria*	GAGAGGAAGGTCCCCCAC	
AllBac412r	(general)	CGCTACTTGGCTGGTTCAG	
BAC-338f	*Bacteroides*	ACTCCTACGGGAGGCAG	
BAC-805R		GACTACCAGCGTATCTAATCC	
sg-Clept-F	*Clostridium*	GCACAAGCAGTGGAGT	
sg-Clept-R	cluster IV	CTTCCTCCGTTTTGTCAA	
g-Ccoc-F	*Clostridium*	AAATGACGGTACCTGACTAA	Matsuda *et al*.,
g-Ccoc-R	*coccoides* group	CTTTGAGTTTCATTCTTGCGAA	2009
sg-Clept-F	*Clostridium*	GCACAAGCAGTGGAGT	
sg-Clept-R	*leptum* subgroup	CTTCCTCCGTTTTGTCAA	
g-Bfra-F2	*Bacteroides*	AYAGCCTTTCGAAAGRAAGAT	
g-Bfra-R	*fragilis* group	CCAGTATCAACTGCAATTTTA	
g-Bifid-F	*Bifidobacterium*	CTCCTGGAAACGGGTGG	
g-Bifid-R	*bifidum*	GGTGTTCTTCCCGATATCTACA	
BiBRE-1	*Bifidobacterium*	CCGGATGCTCCATCACAC	
BiBRE-2	*breve*	ACAAAGTGCCTTGCTCCCT	
c-Atopo-F	*Atopobium*	GGGTTGAGAGACCGACC	
c-Atopo-R	*cluster*	CGGRGCTTCTTCTGCAGG	
g-Prevo-F	*Provetella*	CACRGTAAACGATGGATGCC	
g-Prevo-R		GGTCGGGTTGCAGACC	
CIPER-F	*Clostridium*	GGGGGTTTCAACACCTCC	
CIPER-R	*perfingens*	GCAAGGGATGTCAAGTGT	
En-lsu3F	*Enterobacteria-*	TGCCGTAACTTCGGGAGAAGG	
	ceae	CA	
En-lsu3’R		TCAAGGACCAGTGTTCAGTGTC	
sg-Lcas-F	*Lactobacillus*	ACCGCATGGTTCTTGGC	
sg-Lcas-R	*casei* subgroup	CCGACAACAGTTACTCTGCC	

Table 5 (Continued)

Primer	Target	Sequence (5′ → 3′)	Reference
sg-Lgas-F	*Lactobacillus*	GATGCATAGCCGAGTTGAGAGACTGA	Matsuda
	gasseri	T	*et al*.,
sg-Lgas-R	subgroup	TAAAGGCCAGTTACTACCTCTATCC	2009
sg-Lpla-F	*Lactobacillus*	CTCTGGTATTGATTGGTGCTTGCAT	
sg-Lpla-R	*plantarum*	GTTCGCCACTCACTCAAATGTAAA	
	subgroup		
sg-Lreu-F	*Lactobacillus*	GAACGCAYTGGCCCAA	
sg-Lreu-R	*reuteri* subgr.	TCCATTGTGGCCGATCAGT	
sg-Lrum-F	*Lactobacillus*	CACCGAATGCTTGCAYTCACC	
sg-Lrum-R	*ruminis*	GCCGCGGGTCCATCCAAAA	
sg-Lsak-F	*Lactobacillus*	CATAAAACCTAMCACCGCATGG	
sg-Lsak-R	*sakei* subgroup	TCAGTTACTATCAGATACRTTCTTCTC	
s-Lbre-F	*Lactobacillus*	ATTTTGTTTGAAAGGTGGCTTCGG	
s-Lbre-R	*brevis*	ACCCTTGAACAGTTACTCTCAAAGG	
LFer-1	*Lactobacillus*	CCTGATTGATTTTGGTCGCCAAC	
LFer-2	*fermentum*	ACGTATGAACAGTTACTCTCATACGT	
s-Lfru-F	*Lactobacillus*	TGCGCCTAATGATAGTTGA	
s-Lfru-R	*Fructivorans*	GATACCGTCGCGACGTGAG\	
g-Encoc-F	*Enterococcus*	ATCAGAGGGGGATAACACTT	
g-Encoc-R		ACTCTCATCCTTGTTCTTCTC	
g-Staph-F	*Staphylococcus*	TTTGGGCTACACACGTGCTACAATGGA	
		CAA	
g-Staph-R		AACAACTTTATGGGATTTGCWTGA	
PSD7F	*Pseudomonas*	CAAAACTACTGAGCTAGAGTACG	
PSD7R		TAAGATCTCAAGGATCCCAACGGCT	
En-lsu3’R	*Enterobacteria*	TGCCGTAACTTCGGGAGAAGGCA	
Ec-ssu1F	*-ceae*	TCAAGGACCAGTGTTCAGTGTC	
Ec-ssu1F	*Enterococcus*	TGCCGTAACTTCGGGAGAAGGCA	
Ec-ssu1R		TCAAGGACCAGTGTTCAGTGTC	

Table 5 (Continued)

Primer	Target	Sequence (5′→ 3′)	Reference
STPYF	*Staphylococcus*	ACGGTCTTGCTGTCACTTATA	Matsuda
STPYR2		TACACATATGTTCTTCCCTAATAA	*et al*., 2007
PSD7F	*Pseudomonas*	CAAAACTACTGAGCTAGAGTACG	
PSD7R		TAAGATCTCAAGGATCCCAACGGC T	
CIPER-F	*Clostridium*	AGATGGCATCATCATTTCAAC	
CIPER-R	*perfingens*	GCAAGGGATGTCAAGTGT	
Bif-F	*Bifidobacterium*	TCGCGTC(C/T)GGTGTGAAAG	Songjinda
Bif-R		CCACATCCAGC(A/G)TCCAC	*et al*., 2007
LactoR'F	*Lactobacilli*	CACAATGGACG(A/C)AAGTCTGAT G	
LBFR		CGCCACTGGTGTTCTTCCAT	
BacP-F	*Bacteroidaceae*	GGTGTCGGCTTAAGTGCCAT	
BacP-R		CGGA(C/T)GTAAGGGCCGTGC	
Ecol457F	*Enterobacteriac eae*	CATTGACGTTACCCGCAGAAGAAG C	
Ecol652R	*Enterococci*	CTCTACGAGACTCAAGCTTGC	
Enc-F		CCCTTATTGTTAGTTGCCATCATT	
Enc-R		ACTCGTTGTACTTCCCATTGT	
Cl-per-F	*Clostridium perfringens*	ATGCAAGTCCG(A/C)AAGTCTGAT G	
Cl-per-R	group	TATGCGGTATTAATCT(C/T)CCTTT	
g-Bifid-F	*Bifidobacte-*	CTCCTGGAAACGGGTGG	Matsuki
g-Bifid-R	*rium*	GGTGTTCTTCCCGATATCTACA	*et al*., 2004
BiADOg-1a	*B. adolescentis*	CTCCAGTTGGATGCATGTC	
BiADOg-1b	group	TCCAGTTGACCGCATGGT	
BiADO-2		CGAAGGCTTGCTCCCAGT	
BiANG-1	*B. angulatum*	CAGTCCATCGCATGGTGGT	
BiANG-2		GAAGGCTTGCTCCCCAAC	

Table 5 (Continued)

Primer	Target	Sequence (5′→ 3′)	Reference
BiBIF-1	*B. bifidum*	CCACATGATCGCATGTGATTG	Matsuki
BiBIF-2		CCGAAGGCTTGCTCCCAAA	*et al*., 2004
BiBRE-1	*B. breve*	CCGGATGCTCCATCACAC	
BiBRE-2		ACAAAGTGCCTTGCTCCCT	
BiCATg-1	*B. catenulatum*	CGGATGCTCCGACTCCT	
BiCATg-2	group	CGAAGGCTTGCTCCCGAT	
BiLON-1	*B. longum*	TTCCAGTTGATCGCATGGTC	Matsuki
BiLON-2		GGGAAGCCGTATCTCTACGA	*et al*., 2002
BiINF-1	*B. infantis*	TTCCAGTTGATCGCATGGTC	
BiINF-2		GGAAACCCCATCTCTGGGAT	
BiDEN-1	*B. dentium*	ATCCCCGGGGGTTCGCCT	
BiDEN-2		GAAGGGCTTGCTCCCGA	
g-Bfra-F	*Bacteroides*	ATAGCCTTTCGAAAGRAAGAT	
g-Bfra-R	*fragilis* group	CCAGTATCAACTGCAATTTTA	
g-Prevo-F	*Provetella*	CACRGTAAACGATGGATGCC	
g-Prevo-R		GGTCGGGTTGCAGACC	
g-Bifid-F	*Bifidobacterium*	CTCCTGGAAACGGGTGG	
g-Bifid-R		GGTGTTCTTCCCGATATCTACA	
g-Ccoc-F	*Clostridium*	AAATGACGGTACCTGACTAA	
g-Ccoc-R	*coccoides* group	CTTTGAGTTTCATTCTTGCGAA	

Sequence in IUPAC code; R = G/A, W = A/T, Y= T/C

5.2.6 Terminal Restriction Fragment Length Polymorphism (T-RFLP)

Terminal Restriction Fragment Length Polymorphism (T-RFLP) is a type of Restriction Fragment Length Polymorphism (RFLP). It is based on the restriction endonuclease digestion of fluorescently end-labelled PCR products. The forward primers are usually labled with 6-carboxyfluorescein (6-FAM). The digested products are separated by gel electrophoresis and detected on an automated sequence analyzer (Figure 2). The length of each fragment was compared with reference strains. The discrimination down to strain

level can be reached although the number of fragments makes the interpretation of the profiles difficult. The method is quick and provides distinct profiles (fingerprints) dependent on the species composition of the communities of the samples (Cocolin and Ercolini, 2008; Fernàndez-Guerra *et al*., 2012).

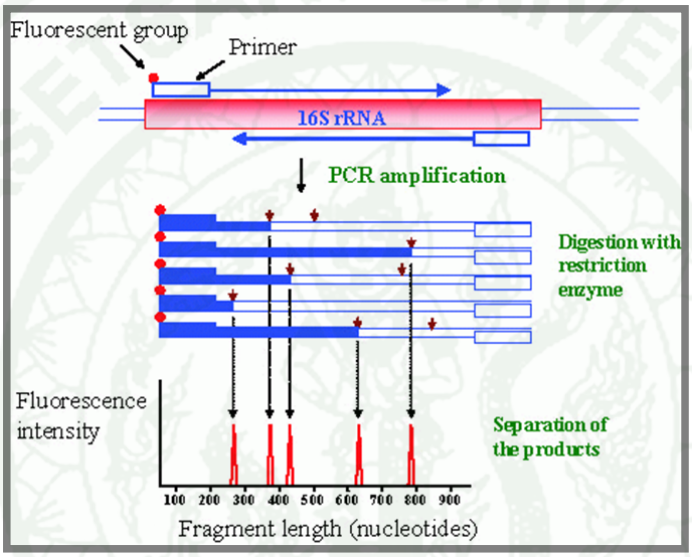

Figure 2 Principle of T-RFLP

Source: Fernàndez-Guerra *et al*. (2012)

T-RFLP was used to analyze for complex bifidobacterial communities in three human fecal samples using *Bifidobacterium* genus-specific PCR primers. They could identify more than one bifidobacterial species. T-RFLP pattern of *Bifidobacterium* genus-specific PCR products were host-specific as well as those of 16S rDNA PCR products. Each of three patterns showed 34-50 terminal restriction fragments (T-RFs) after digestion with *Msp*I. The size of the majority of *Msp*I-digested T-RFs was less than 600 bp. The results were confirmed by PCR-DGGE with primer specific for the genus *Bifidobacterium* and *Bifidobacterium* species- and group-specific PCR. This study demonstrates that T-RFLP analysis is useful for assessment of the diversity of the human fecal microbiota and rapid comparison of the community structure among individuals (Sakamoto *et al*., 2002).

Fecal samples from 6 elderly subjects were characterized by 16S rDNA libraries and T-RFLP analysis for microbiota study. The 16S rDNA libraries were

derived from 3 elderly individuals after PCR amplification with universal primer set. After digestion by *Hha*I and *Msp*I, T-RFLP analysis was performed using amplified from 6 individual. The 16S rDNA libraries and T-RFLP analysis revealed that the majority of bacteria were *Bacteroides* and relatives, *Clostridium* rRNA cluster IV, IX, *Clostridium* rRNA subcluster XIVa and *Gammaproteobacteria*. *Gammaproteobacteria* were detected at high frequency. The lengths of T-RFs were in Table 6 (Hayashi *et al*., 2003).

Table 6 The length of terminal restriction fragment after digestion by *Hha*I and *Msp*I

Bacteria	length of T-RF digested by *Hha*I (bp)	length of T-RF digested by *Msp*I (bp)
Bacteroides and relatives	100	90
Clostridium rRNA cluster IV	35	290
Clostridium rRNA subcluster XIVa	1,070	200
Gammaproteobacteria	370	490

Source: Hayashi *et al*. (2003)

Autopsies of three elderly individuals in gut contents of jejunum, ileum, caecum and recto-sigmoid colon were collected to investigate their intestinal microbiota. The 16S rDNA libraries and T-RFLP digestion with *Hha*I and *Msp*I were used in this study. The microbiota of jejunum and ileum consisted of simple microbial communities of streptococci, lactobacilli, *Gammaproteobacteria*, the Enterococcus group and *Bacteroides* group. The *Clostridium coccoides* group and the *Clostridium leptum* subgroup were not detected in samples from the upper gastrointestinal tract. The microbiota of caecum was more complex than those of the jejunum and ileum. The *C. coccoides* group, the *C. leptum* subgroup and *Bacteroides* group were detected in the caecum. The recto-sigmoid colonic microbiota consisted of complex microbial communities. The numerous species belonged to the *C. coccoides* group, the *C. leptum* subgroup and *Bacteroides* group, *Gammaproteobacteria*, the *Bifidobacterium* group, streptococci, lactobacilli (Hayashi *et al*., 2005).

Matsumoto *et al*. (2005) used T-RFLP to build a phylogenetic assignment database for human colonic microbiota (PAD-HCM) by T-RFLP analysis and to

demonstrate effectiveness of PAD-HCM compared with the result of 16S rRNA gene clone library analysis. PAD-HCM was completed to include 342 sequence data obtained using four restriction enzymes (*Hha*I, *Msp*I, *Hae*III and *Alu*I). Aproximately 80% of the total clones detected by16S rRNA gene clone library analysis were the same bacterial species or phylotypes as those assigned from T-RF using PAD-HCM. All pseudo-T-RFs identified by mung bean nuclease digestion could not be assigned to a bacterial species or phylotype. They concluded that PAD-HCM built in this study enabled to predict T-RFs at the species level including difficult-to culture bacteria. It is very useful for the RFLP analysis of human colonic microbiota.

Genomic DNA was extracted from fecal slurries. The bacterial 16S rDNA gene was amplified and analyzed by T-RFLP. This research tested the effects of experimental conditions on DNA quality, DNA quantity, and T-RFLP pattern derived from gut bacterial communities. The result showed that the composition of T-RFLP fingerprints varied by different extraction procedure. The best quality and quantity of community DNA extracted from fecal sample was obtained by using the QIAamp DNA stool minikit (Qiagen, CA, USA) with 95°C incubation and moderate bead beating during the cell-lysis step. The researchers assessed the methodological and inter-individual variation in gut community fingerprints. They concluded that standardized T-RFLP is a robust, reproducible and high throughput method. It will provide a useful biomarker for characterizing gut microbiota in human fecal samples (Li *et al.*, 2007).

T-RFLP has efficiency, reliability and high reproducibility. It contains ability to assess a direct phylogenetic affiliation of each member within the community. T-RFLP analysis could not be limited to ribosomal gene markers. The main limitations of T-RFLP are fairly high cost and time-consuming (Cocolin and Ercolini, 2008).

5.2.7 Pyrosequencing

Pyrosequencing is a DNA sequencing technology based on the sequencing-by-synthesis principle. This technique was developed by Pål Nyrén and his student Mostafa Ronaghi at the Royal Institute of Technology in Stockholm in 1996 (Wikipedia 2012a, Pyrosequencing, retrieved on Mar 14, 2012). It relies on real-time detection of pyrophosphate (PPi) release on nucleotide incorporation rather than chain termination with dideoxynucleotides. A pyrosequencing cycle takes about one minute and

involves a cascade of four enzymes reactions on a real-time monitoring of DNA synthesis by bioluminescence. The four enzymes included in pyrosequencing system are the Klenow fragment of DNA polymeraseI, ATP sulfurylase, luciferase and apyrase. The reaction mixture also contains the enzyme substrates adenosine phosphosulfate (APS), D-luciferin and the template with annealed primer to be used as starting material for DNA polymerase. The four deoxynucleotide triphosphate molecules (dNTP) are added one at a time. The first reaction, the DNA polymerase I, included in the reaction mixture to catalyse the reaction. All template molecules can be achieved. A misincorporation rate of less than 10^{-5}-10^{-6} is achieved even without the proofreading (3′ to 5′ exonuclease). The PPi quickly released during incorporation of a nucleotide reacts to produce ATP (adenosine triphosphosfate) in a reaction catalysed by ATP sulfurylase. On the other hand, any of non-complementary nucleotides would not release PPi. The ATP sulfurylase used in pyrosequencing is a recombinant enzyme from *Saccharomyces cerevisiae*. The generated ATP is used as energy source in luciferase (from firefly insect) oxidizing luciferin reaction and in the process generated light. This amount of light is easily detected by a photodiode, photomultiplier tube, or a CDD-camera (Charge Coupled Device camera). The final enzymatic reaction is catalysed by apyrase which a nucleotide degrading enzyme from potato. Low amont of this enzyme in the reaction mixture efficiently degrade unincorporated dNTP and do not use ATP. All reactions of pyrosequencing are show in 1-6 equations.

$$(DNA)_n + dNTP \xrightarrow{\text{polymerase}} (DNA)_{n+1} + PPi \quad (1)$$

$$PPi + APS \xrightarrow{\text{ATP sulfurylase}} ATP + SO_4^{2-} \quad (2)$$

$$\text{Luciferase} + \text{D-luciferin} + ATP \longrightarrow \text{Luciferase-D-luciferin-AMP} + PPi \quad (3)$$

$$\text{Luciferase-D-luciferin-AMP} + O_2 \longrightarrow \text{Luciferase} + \text{oxyluciferin} + AMP + CO_2 + \text{light} \quad (4)$$

Apyrase removed unincorporated nucleotides and ATP between the additions of different bases.

$$ATP \xrightarrow{\text{apyrase}} AMP + 2Pi \quad (5)$$

$$dNTP \xrightarrow{\text{apyrase}} dNMP + 2Pi \quad (6)$$

The entire process from polymerization to light detection takes 3-4s at room temperature. In the present of one pmol template, one pmol nucleotide incorporation yields 6×10^{11} ATP molecules, which in turn, generate about 6×10^{9} photons at a wavelength of 560 nm (Rapley and Habron, 2004; Ahmadian *et al*., 2006).

Since complex microbial communities of human believed to contribute to health maintenance, determination of microbial composition in patients and healthy controls may provide novel therapeutic information. Pyrosequencing technique was used to analyze human gut microbiota. Andersson *et al*. (2008) applied this technique to analyze microbial communities in throat, stomach and fecal samples. In case of stomach, microbial communities in *Helicobacter pylori*-negative stomach and *Helicobacter pylori*-positive stomach were compared. The throat microbiota displayed the lowest phylotype of the three ecosystems. There were 8 genera (*Streptococcus, Prevotella, Actinomyces, Gemella, Rothia, Granulicatella, Haemophilus and Veillonella*) present in all of throat samples and in the previously reported esophagus samples. *Streptococcus* was the dominant genus followed by *Prevotella.* In *H. pylori*-negative stomach of three samples were found *Streptococcus, Actinomyces, Prevotella* and *Gemella* as prominent phylotypes. Firmicutes and Bacteroides are dominated in the fecal samples. Firmicutes dominated in the six fecal samples. The majority of the class Clostridia belonging to the Firmicutes frequently represented the genera *Ruminococcus*, *Clostridium* and *Eubacterium*. It was surprised that Actinobacteria was the second most abundant phylum which belonging to genus *Bifidobacterium* and to the family Coriobacteriaceae in all samples. The Bacteroidetes were dominated by various *Bacteroides* phylotypes. Their results demonstrate the applicability of pyrosequencing as a high-throughput method for comparative microbial ecology.

The gut microbiota of 20 Koreans were investigated using 454-pyrosequencing with barcode primers. It was found that common 5 phyla encountered in the intestine, Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria and Proteobacteria. The Firmicutes (70.8% of reads on average) and Bacteroidetes (24% of reads on average) were predominants. The three most abundant genera were *Faecalibacterium* (10% of reads), *Prevotella* (9.5% of reads) and *Bacteroides* (8.9% of reads) (Nam *et al*., 2011).

This technique advantageously compared to other sequencing methods of applications. The results could be reported at the species level. They would

show the relative community of each sample as well. It is powerful as Sanger sequencing with high discrimination level, reproducibility, accuracy, easy interpretation and rapidity. Its limitations are very expensive, requirement of experiance technique (Rapley and Habron, 2004; Ahmadian *et al*., 2006; Andersson *et al*., 2008).

5.2.8 Illumina Genome Analyzer

This method is being in the next generation technique for sequencing as the same as pyrosequencing based on the sequencing-by-synthesis principle as same as pyrosequemcing. The preparation of the sequencing library is done by bridge PCR, while the sequencing is done by a technology referred to cyclic reversible termination. It is not used for microbiota analyzing yet now, but it can be applied for genome sequencing (Ahn *et al*., 2009), cancers screening, including colorectal cancer (Link *et al*., 2010), and prostate cancer screening (Eles *et al*., 2008).

After genomic DNA shearing, the single molecule amplification step for the Illumina Genome Analyzer starts with an Illumina-specific adapter library. It takes place on the oligo-derivatized surface of a flow cell. It is performed by an automated device called a cluster station. The flow cell is an eight-channel sealed glass microfabricated device that allows bridge amplification of fragments on its surface (Figure 3). This system uses DNA polymerase to produce multiple DNA copies, or clusters. Each copy represents the single molecule that initiated the cluster amplification. The Illumina system utilization, all four fluorescently labelled 3′-OH blocked nucleotides are added to the flow cell with DNA polymerase for incorporation into the oligo-primed cluster fragments (Figure 4). The cluster strands are extended by one nucleotide. So, each of incorporation is a unique event. Following the incorporation step, the unused nucleotides and DNA polymerase molecules are washed away by a scan buffer which is added to the flow cell. The optics system scans each lane of the flow cell by imaging units called tiles. An imaging step follows each base incorporation step, during which each flow cell lane is imaged in three 100-tile segments by the instrument optics at a cluster density per tile of 30,000. After each imaging step, the 3′-OH blocking group is chemically removed to prepare each strand for the next incorporation by DNA polymerase. This series of steps continues for a specific number of cycles, as determined by user-defined instrument settings, which permits discrete read lengths of 25–35 bases. A base-calling algorithm assigns sequences and associated quality values to each read and a quality checking pipeline evaluates the Illumina data from each run,

removing poor-quality sequences (Mardis 2008).

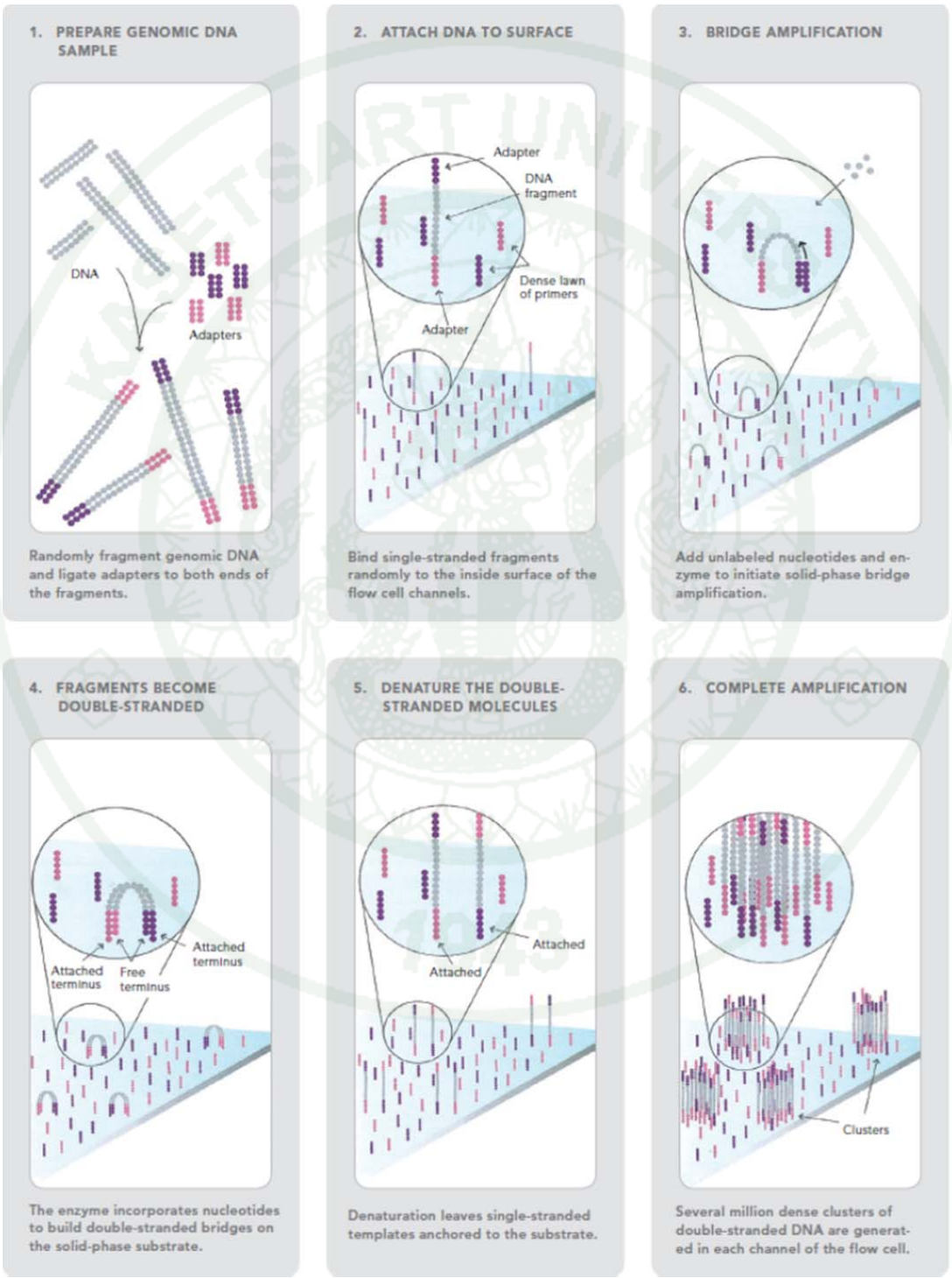

Figure 3 Illumina HiSeq 2000 steps (Part I)

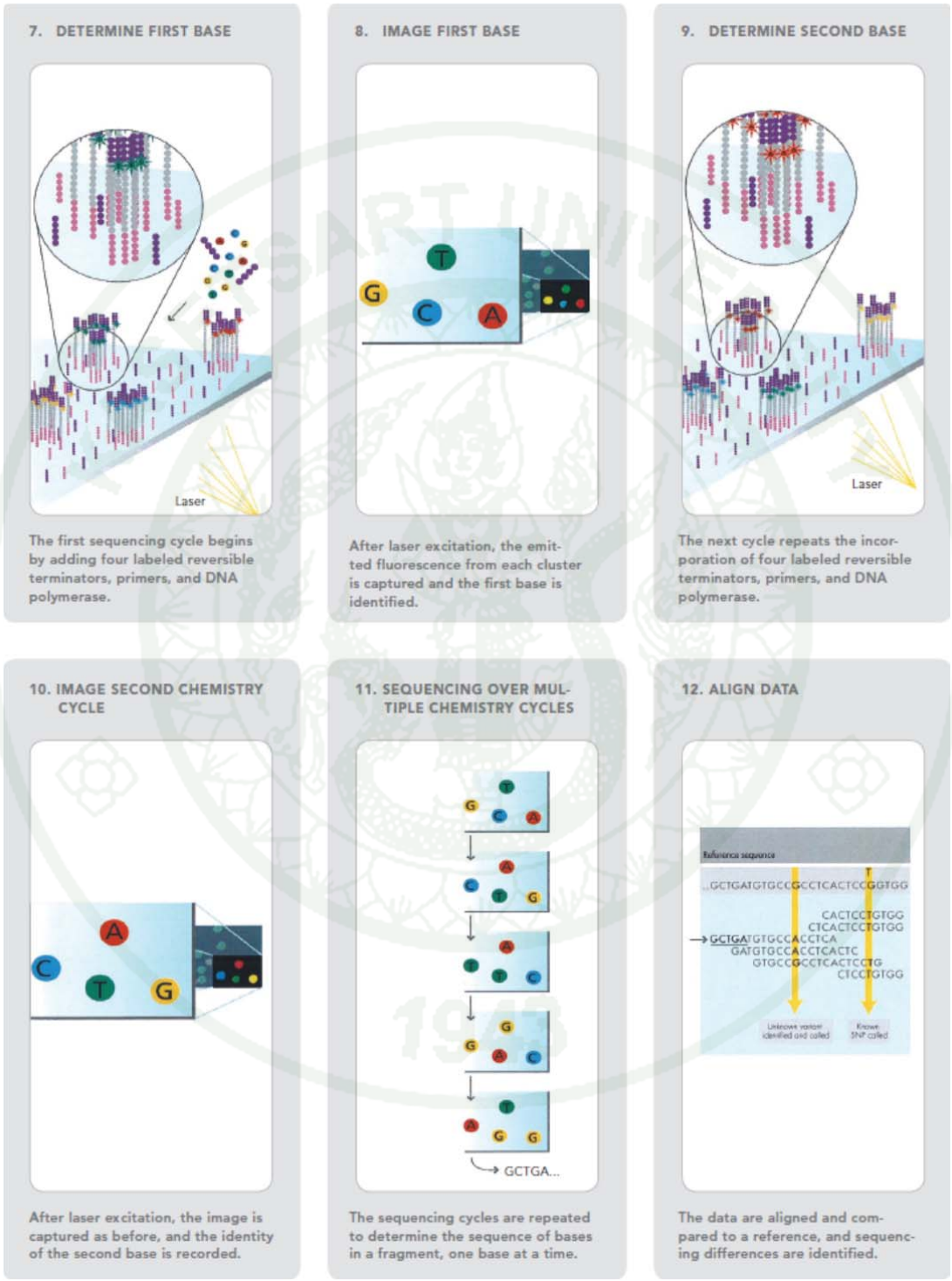

Figure 4 Illumina HiSeq 2000 steps (Part II)

The summary of the most widely used techniques for human microbiota was shown on Table 7.

Table 7 Summary of the most widely used techniques for human microbiota

Technique	16S rRNA cloning	FISH (Non-culture)	DGGE & TGGE	T-RFLP	qPCR	Pyro-Sequen-cing
Time-Consuming	Fair	Rapidity	Fair	Fair	Rapidity	Rapidity
Cost	Low	High	Moderate	High	High	Very high
Discrimination	Fair	Moderate	Moderate to high	High	Fair	High to excellent
Reproducibility	Best/ Good	Moderate	Best/ Good	Moderate	Best/ Good	High
Interpretation	Easy	Easy	Moderate	Easy	Easy	Easy
Public data available	✓	X	✓	✓	✓	✓
Taxonomic resolution	Genus / species level	Species Level	Species / strain level	Species / strain level	Genus / species level	Species / strain level
Easy to use	Easy	Easy	Easy	Moderate	Moderate	Easy

✓; yes and x ; no

Source: Tannock (1999); O'Sullivan (2000); Vanughan *et al*. (2000); Rapley and Habron (2004); Ahmadian *et al*. (2006); Rajilić-Stojanović *et al*. (2007); Cocolin and Ercolini (2008)

MATERIALS AND METHODS

Materials

1. Equipments

1.1 Autoclave (Hirayama HV 25, Hirayama)

1.2 Balance (Satorius A200S)

1.3 Centrifuge, refrigerated microcentrifuge (Mikro 200 R)

1.4 Centrifuge tube (50 ml sterile centrifuge tube with screw cap)

1.5 Cotton swab: Sterile cotton swab (Thai Gauze)

1.6 Disposable tissue papers **(**Folded paper towel, Scott Kimberly-Clark)

1.7 Dry block bath (Boekel)

1.8 Electrophoresis unit (DGGE, The DCode™ Universal Mutation Detection system)

1.9 Gel documentation system (Syngene)

1.10 Gradient former (Model 485, Bio-Rad)

1.11 Hot air oven (Memmert)

1.12 Incubator water bath (Memmert)

1.13 Micropipette (Gilson and Eppendorf)

1.14 Microcentrifuge tubes (1.5 and 2 ml microcentrifuge tube, Axygen)

1.15 Minicentrifuge (C-1200, Labnet)

1.16 Peristatic pump (Pump P-1, Phamacia Biotech)

1.17 Power supply (POWER PAC 300, Bio-Rad)

1.18 Real-time PCR (LightCycler® LC480, Roche)

1.19 Sequencer (Genome sequencer FLX454 Titanium System, 454 Life Sciences)

1.20 Spectrophotometer (Nanodrops 2000c, Thermo Fisher Scientific)

1.21 Thermocycler (Biometra Tgradient, Biometra)

1.22 Vortex mixer (Kika-works MS1)

2. Chemicals

2.1 Absolute ethanol (Merck, Germany)

2.2 Acrylamide (Bio-Rad, USA)

2.3. Agarose (molecular biology grade, Vivantis, Vivantis Technologies Sdn. Bhd., Malaysia)

2.4 Ammonium persulfate (Bio-Rad, USA)

2.5 Bis-acrylamide (Bio-Rad, USA)

2.6 Cloning system (pGEM-T Easy vector kit, Promega, USA and RBC T&A Cloning Vector Kit, RBC Bioscience Corporation, Taiwan)

2.7 DNA extraction kit (QIAamp® Stool Mini Kit, QIAGEN, Germany)

2.8 DNA polymerase kit **(**TAKARA Ex *Taq* Kit, TAKARA, Japan and LightCycler® 480 SYBR Green I Master, Roche, Germany)

2.9 DNA purification kit (QIAamp DNA purify kit, Qiagen, Germany and GENEAll® DNA Purification System, Geneall Biotechnology, Korea)

2.10 DNA marker: The 100 bp plus, Fermentas, Canada

2.11 Ethylenediamine tetraacetic acid (EDTA): Sigma, USA

2.12 Formamide (deionized): Amersco, USA

2.13 Glacial acetic acid: Merck, Germany

2.14 Hydrochloric acid: Merck, Germany

2.15 Nucleic acid staining (GelStar™ Nucleic acid gel stain, Lonza, USA and SYBR® Gold Nucleic acid gel stain, Invitrogen, USA)

2.16 *N,N,N′,N′*-tetramethylethylene diamine (TEMED) (Bio-Rad, USA)

2.17 Transfomation system (RBC Hit Competent Cell Kit, RBC Bioscience Corporation, Taiwan)

2.18 Tris (hydroxymethyl) aminonethane (Sigma, USA)

2.19 Urea (molecular biology grade, Scharlau Chemie, Scharlab, Spain)

2.20 Zirconium beads (0.1 mm, Bio Spec Products, USA)

Methods

1. Faecal sampling for gut microbiota investigation

Out of 72 faecal samples used in this study, 36 samples of vegetarians aged between 40 and 61 years (subject V1 to V36) and 36 samples from non-vegetarians (subject N1 to N36) aged between 41 and 78 years, were collected. Most of vegetarian volunteers live and work at the famous Thai vegetarian society in Bangkok called Santi-Asoke. They were ovo-lacto-, lacto- or ovo-vegetarians or vegans and 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 (IBD), no diarrhea in the month preceding the sampling, and no family history of colorectal cancer. None of the subjects had received any antibiotic treatment within at least one month prior to this study. A faecal 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 (Figure 5 and Appendix A to C). Each subject would be collected his faecal sample by himself according to the instruction provided in a faecal sampling kit. In brief, the provided sterile disposable tissue papers were laid on the dried area for excretion. The faecal samples were carefully swabbed by sterile cotton swabs and applied into the bottom of sterile tube which tightly closed screw cap before sealing in a plastic bag. All samples were kept on ice when they were transported to laboratory and maintained at -80°C till required.

2. Questionnaire and consent form

Each subject would be informed to carefully read consent form and the personal information in the questionnaire. All documents were presented in Appendix A to C. The study protocol and consent documents were approved by the Institute for the Development of Human Research Protections (IHRP) under ethic approval No. IHRP 311 (Appendix D).

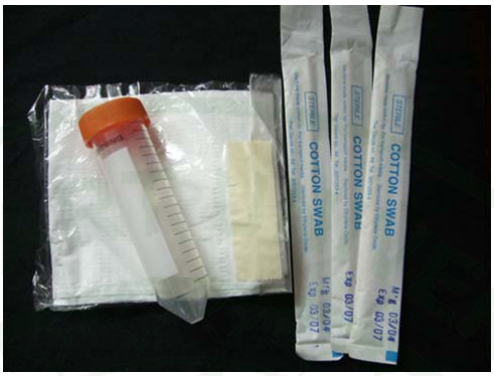

Figure 5 Faecal sampling kit

3. DNA extraction

Each faecal sample from stool sampling tube was aliquot 40-60 mg into new 1.5 ml sterile microcentrifuge tubes per tube. It was washed two times with 1 ml of Tris-buffer, pH 8 containing 20 mM Tris-HCl and 2 mM EDTA. The samples were settled by centrifugation at 8,000 rpm for 10 min at room temperature and supernatant was discarded. Each faecal sample was homogenized in buffer ASL 1.4 ml of QIAamp® Stool Mini Kit from QIAGEN with 0.3 g of 0.1 mm sterile zirconium beads by vortexing at 2,000 rpm for 1 min followed by keeping on ice for 1 min. This step was repeated 3 times. Thereafter, the total bacterial genomic DNA was extracted according to the kit instructions. Briefly, each stool suspension was heated at 95 °C for 5 min and mixed by vortexing for 15 sec. To pellet stool particles, all samples were settled by centrifugation at 14,000 rpm for 1 minute. The supernatant was transferred to new 2 ml sterile microcentrifuge tube. The pellet was discarded. An InhibitEX Tablet was added to each sample and mixed immediately for 1 min before incubation at room temperature for 1 min to allow adsorption of inhibitors to InhibitEX matrix. After that, the suspension was settled by centrifugation at 14,000 rpm for 3 min and supernatant was transferred to a new 1.5 ml sterile microcentrifuge tube. This step was repeated once more. The supernatant was transferred to 1.5 ml sterile microcentrifuge tube containing 30 µl of proteinase K and mixed by vortexing. Four hundred microliter of buffer AL were then added into the tube and mixed by vortexing. After incubation at 70 °C for 10 min, 400 µl of absolute ethanol were added and mixed by vortexing. The solution was then transferred to the QIAamp spin column and centrifuged at 8,000 rpm for 1 min. The filtrate was discarded. Each spin column was washed with 500 µl of buffer AW1 and

centrifuged at 8,000 rpm for 1 min. The spin column was washed again with 500 µl of buffer AW2. To completely dry the spin column, centrifugation was carried out again at 8,000 rpm for 3 min. The DNA was eluted from the spin column with 50 µl of sterilized pure water and kept at -20°C until use. Both quality and quantity of the extracted DNA were determined by spectrophotometer.

4. PCR-DGGE of 16S rDNA

This method was used for preliminary investigation of gut microbiota. Only 13 samples, 7 samples of vegetarian group (subject V1 to V7) and 6 samples of non-vegetarian group (subject N1 to N6) were used in this step. Two primers, namely HDA1-GC and HDA2 (Songjinda *et al*., 2005), were used to amplify V2-V3 region of the 16S rRNA gene of each sample. The sequences for the primers were as follows: HDA1-GC, 5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG T-3' and HDA2, 5'-GTA TTA CCG CGG CTG CTG GCA C-3'. These primers were used to amplify V2-V3 region of the 16S rRNA gene sequences. For each sample, PCR mixture containing 1x Ex *Taq* buffer, 0.2 mM of each dNTP, 0.4 µM of each primer and 1.25 U of Ex *Taq* DNA polymerase (TAKARA Ex Taq Kit) was prepared. Fifty nanogram of the extracted template DNA was added into each reaction. The PCR-DGGE conditions were as follows: 94°C for 5 min, 30 cycles at 94°C for 40s, 58°C for 20s, and 72°C for 1min, and a final elongation at 72°C for 5 min (Songjinda *et al*., 2005). The expected size of PCR product was 250 bp. The 100 bp plus were used as DNA marker. The amplified 16S rDNA genes were purified using QIAamp DNA purify kit. The PCR-DGGE profile was performed using a Dcode System apparatus from BioRad (California, USA). The electrophoresis was run using a 25-65% denaturing gradient with 100% corresponding to 7M urea and 40% formamide in an 8% polyacrylamide gel, at 80 volts for 1 hour and subsequently at 100 volts for 6 hours at 60°C.

After the PCR-DGGE running step, a dendrogram was constructed to cluster the PCR-DGGE profile. The similarities between the PCR-DGGE profiles were analyzed based on the locations of the DNA bands on the PCR-DGGE gel using SYNGENE Gene Tools version 4.03(b) and SYNGENE Gene Directory Application version 2.01 (c) from SYNGENE, a division of Synoptic Ltd., using Dice's similarity coefficient and an unweighted pair group method with arithmetic mean (UPGMA) algorithm .

To determine the gut microbiota using PCR-DGGE, the bands of interest were cut and eluted with sterile pure water. Each eluted band was then re-amplified with the HDA1-GC and HDA2 primers and run on the DGGE system at a suitable gradient concentration to check the purity of the cut band. Following the purity check, each band was re-amplified without a GC clamp and then purified using a QIAquick PCR Purification kit (QAIGEN). The correct target size (approximately 200 bp) was confirmed on 2% agarose gel electrophoresis. The purified PCR products were subsequently analyzed using a direct sequencing analysis performed by 1st BASE, Malaysia. The fragment of interest was identified using BLAST in the NCBI and Eztaxon databases.

5. Quantitative real-time PCR analysis

This step was optimized for interested specific groups including *Prevotella, Bacteroides* (based on the *Bacteroides fragilis* group), *Bifidobacterium*, *Lactobacillus, Clostridium coccoides-Eubacterium rectale* group (*Clostridium* subcluster XIVa), *Clostridium leptum* group (*Clostridium* cluster IV), Enterobacteriaceae, and *Faecalibacterium prausnitzii-Subdoligranulum variabile* like bacteria.

5.1 DNA standard used for real-time PCR

Eight bacterial groups in each subject were quantified using quantitative real-time PCR (LightCycler®LC480, Roche, the Netherlands). Specific primers for *Prevotella*, *Bacteroides*, *Bifidabacterium*, *C. leptum* group, *C. coccoides-E. rectale* group, Enterobacteriaceae, *Lactobacillus* and *F. prausnitzii-S. variabile* like bacteria were shown in Table 8. The extracted genomic DNA from *Prevotella nigrescenes* JCM 12250T, *B. fragilis* ATCC 25285, *Bifidobacterium bifidum* JCM 1255, *Blautia producta* JGD 07421, *C. leptum* DSM 753, *Salmonella* Typhimurium TISTR 292, *Lactobacillus casei* subsp. Rhamnosus ATCC 7469 and *F. prausnitzii* DSM 17677 were used to perform the standard curve of each specific group of *Prevotella*, *Bacteroides*, *Bifidabacterium*, *C.coccoides-E. rectale*group, *C. leptum* group, Enterobacteriaceae, *Lactobacillus,* and *F. prausnitzii-S. variabile* like bacteria, respectively.

Each standard curve was constructed using specific primers to amplify the reference bacteria as mention above. PCR products of *P. nigrescenes* JCM 12250T, *B. fragilis* ATCC 25285, *C. leptum* DSM 753 were separately cloned into pGEM-T Easy

vector according to manufacturer's instruction (Promega, USA). On the other hand, PCR products of *L. casei* subsp. Rhamnosus ATCC 7469 and *F. prausnitzii* DSM 17677 was cloned into RBC T&A cloning vector according to manufacturer's instruction (RBC Bioscience Corporation, Taiwan). The other recombinant plasmids; *B. bifidum* JCM 1255, *S.* Typhimurium TISTR 292 and *B. producta* JGD 07421 were constructed by Nakphaichit, 2012. Each recombinant plasmid was diluted by serial 10-fold dilution. The standard curves were generated by amplification of the serial dilution (10^2-10^9 copy number) of each standard DNA of the corresponding bacteria 16S rDNA at least 5 log ranks. The reaction mixture in 20µl of reaction volume contained 10 µl of 2X SYBR Green I master mix from Roche, 2µl of 5µM Forward-Reverse mixed primers set, 6 µl of PCR water and 2 µl of serial dilution standard DNA template. The amplification program consisted of one cycle of 95˚C for 5 min and the 45 cycles of 95˚C for 10 sec, optimized annealing temperatures for each specific group were shown in Table 8 (the extension time was calculated by dividing the target size over 25 according to Roche's recommendation). These standard curves were constructed using LightCycler®LC480 software, second derivative maximum (LightCycler®LC480, Roche, the Netherland). The efficiency of 1.8-2.0 ($E = 10^{-1/slope}$) of standard curve slope is accurate quantification requirement. An acceptable error value should be lower than 0.2 which is a measure of accuracy of the quantification result base on the standard curve. All standard curves were shown in Appendix E.

5.2 Quantification of specific groups using real-time PCR

Fifty to one hundred nanogram of genomic DNA from each subject were used as the template in 20µl of reaction volume as the same as DNA standard. The amplification program was performed using optimized condition. The annealing temperature for each specific group was optimized. PCR products from each sample showed only one targeted size on 2% of agarose gel electrophoresis at the optimized temperature. According to protocol for use with LightCycler® 480 Multiwell Plate 96, the melting curve analysis was carried out by slowly heating the mixed PCR products from 65-97˚C to distinguish targeted PCR products from non-targeted PCR products. The size and purity of targeted PCR products were confirmed by 2% of agarose gel electrophoresis again.

The copy number of DNA fragment was calculated for determination of the number of interested specific species presenting in each sample. Fluorescent signals

detected from DNA fragment were compared to a standard curve generated with standard DNA in the same experiment.

5.3 Specific group primers used for quantification of specific groups of gut microbiota

The specific group primers used in this step, optimal annealing temperature and PCR product size were shown on Table 8.

Table 8 Specific primers used for determination of specific microorganism groups found in human microbiota by real-time PCR

Primer	Target	Sequence (5′→3′)	Target Size (bp)	Annealing temp (°C)	Reference
g-Prevo-F	*Prevotella*	CACRGTAAACGATGGATGCC	513	62	Matsuki
g-Prevo-R		GGTCGGGTTGCAGACC			*et al*., 2002
Bfra-F2	*Bacteroides fragilis*	AYAGCCTTTCGAAAGRAAGTA	495	53	Matsuki
Bfra-R	group	CCAGTATCAACTGCAATTTTA			*et al*., 2002
Bif-F	*Bifidobacterium* sp.	TGGCGTCYGGTGTGAAAG	243	62	Rinttila
Bif-R		CCACATCCAGCRTCC			*et al*., 2004
F_alllact	*Lactobacillus* sp.	TGGATGCCTTGGCACTAGGA	92	55	Haarman and
R_alllact		AAATCTCCGGATCAAAGCTTACTTAT			Knol 2006
Clos-F	*Cl. coccoides*-	CGGTACCTGACTAAGAAGC	429	51	Rinttila
Clos-R	*E. rectale* group	AGTTTYATTCTTGCGAACG			*et al*., 2004
sg-Clept_F	*Clostridium leptum*	GCACAAGCAGTGGAGT	239	50	Matsuki
sg-Clept_R3	*group*	CTTCCTCCGTTTTGTCAA			*et al*., 2004
En-F	*Enterobacteriaceae*	CATTGACGTTACCCGCAGAAGAAGC	195	57	Bartosch
En-R		CTCTACGAGACTCAAGCTTGC			*et al*., 2004

Table 8 (Continued)

Primer	Target	Sequence (5′→3′)	Target Size (bp)	Annealing temp (°C)	Reference
F_Faec	*F. prausnitzii-*	ACCATGAGAGCCGGGGGG	100	57	Lund *et al*.,
PROK1492RT	*S. variabile* group	GGTTACCTTGTTACGACTT			2010

Sequence in IUPAC code; Y = C or T.

6. Pyrosequencing analysis

6.1 Preparation of samples for pyrosequencing process

The 16S rDNA of bacterial DNA was amplified with V6-V8 region specific primer set tagged with barcode sequence, Q-968F-#, 5′CWSWSWWSH WAC GCG ARG AAC CTT ACC3′ and Q-1390R-#, 5′CWSWSWWSHTGA CGG GCG GTG WGT AC3′. # is a series of barcode tags designed by Nakayama. The first nine nucleotides in the sequence were barcode tags (Nakayama, 2010). Each genomic DNA sample approximate 10-100 ng was used as the template in 50 µl of PCR reaction. For each sample, PCR mixture containing 1x 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. PCR condition was as follows: one cycle at 98°C for 2.5 min, 20 cycles at 98°C for 15s, 54°C for 30s, and 72°C for 20s, and a final elongation at 72°C for 5 min. The amplicons were purified using a GENEAll® DNA Purification System (GeneAll, Korea) or QIAquick® 96 PCR purification kit (QIAGEN, USA) according to the manufacturer's protocols. The purified PCR products were determined the DNA concentration using a NanoDrop ND-1000 spectrophotometer. Approximate 100 ng of each purified amplicon from each samples was pooled and purified by ethanol precipitation prior to pyrosequecing process (Nakayama *et al*., 2013). According to the manufacturer's protocol (454 Life Sciences, Roche) the amplicon mixture was applied by emulsion PCR (emPCR) with GS FLX Titanium LV emPCR kit (Lib-L) v2, an amplicon fragment was amplified on a special bead (one fragment one bead). Each amplified DNA-bead was loaded onto a GS FLX Titanium Pico Titer Plate with dividers separated reaction chambers to accommodate 2 mixture pools. The amplified DNA-bead size fixed to a well of the GS FLX Titanium Pico Titer Plate. Pyrosequencing was proceeded using FLX Genome Sequencer (454 Life Sciences) with GS FLX Titanium Sequencing Kit XLR70 according to the manufacturer's protocol.

6.2 Pyrosequencing data analysis

After sequencing process, the 454 batch sequence data were obtained. To acquire each sample batch, the obtained 454 batch data were sorted using the QIIME 1.7.0 software package and other tools. The multiplex reads were attributed to a split_library.py script (QIIME Team 2013b) based on their barcode sequences to generate each sample

sequence data batch. In this step, it achieved quality filtering based on the characteristics of each sequence and removed any low quality or doubtful reads. The parameters used in this script were 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 (Nakayama *et al*., 2013).

Total reads obtained from a set of split_library.py sequences were further filtered their qualities to get rid of noisy sequences, chimera checking and operation taxonomic unit (OTU) clustering via USEARCH v5.2.236 in QIIME (QIIME Team 2013c). The results from this step were obtained when ordered pick_otus.py -i seq.fna -m usearch -f gold.fa -o usearch_qf_result/ --word_length 64 to USEARCH. Ten steps to process the input reads were done in this order. The results were performed as "enumerate_otu.fas" file. The OTUs were calculated to get their IDs replaced by a sequential number. Afterthen, the OTU IDs were picked a representative set of sequences using pick_rep_set.py script at http://qiime.org/scripts/pick_rep_set.html. Each OTU was concluded with one OTU one sequence. To identify the species of each OTU, the phylogenetic composition was determined using the QIIME assign_taxonomy.py script at http://qiime.org/scripts/ assign_ taxonomy.html?highlight=assign_taxonomy). Basic Local Alignment Search Tool (BLAST) alignments were opposited to the Greengenes 13_5 database. Finally, OTU tables were generated using make_otu_table.py and convert_ biom.py.scripts at http://qiime.org/scripts/ make_otu_table.html?highlight=make_otu_table (QIIME Team 2013a).

6.3 Taxonomic analysis

Each OTU sequence was classified to bacterial population data from the phylum to genus (hierarchical level) by the RDP classifier at 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 at http://rdp.cme.msu.edu /seqmath/seqmatch.jsp. which displayed 20 closet 16S rDNA of cultured strains. After seqmqtch process, seqmatch Q400 algorithm (Nakayama, 2010) was used to convert the result of each sample from RDP seqmatch to species level of population. 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 2 species with the same best score, the one with the highest count on the top of 20 lists was selected. The criteria 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 (Nakayama *et al*., 2013).

7. Statistical analysis

Statistical analysis was performed using SPSS statistics software version 18. All data were presented as means ± standard deviations (mean ± SD). Before statistical significance analysis, normality test (measure of dispersion) was performed using one-sample Kolmogorov-Smirnov test. For normal distribution data, differences between sample groups were compared using one-way ANOVA, while Mann-Whitney U test was used for non-normal distribution data. Statistical significance was tested at a *p* values less than 0.05.

Using Multi-Variate Statistical Package (MVSP) version 3.1, the principal component analysis (PCA) was created in order to show differences in bacteria species between both vegetarian and non-vegetarian groups. The first two principal components with high values in PCA were selected to demonstrate the PCA plot as x- and y-axis. The arrow axis indicated key bacterial species related to samples closing to that arrow axis.

Correlation between bacterial species and various factors were calculated using bivariate correlation of SPSS statistics software. For normal distribution data, differences between sample groups were compared using Pearson, while Mann- Spearman test was used for non-normal distribution data. Statistical significance was tested at a *p* values less than 0.05.

RESULTS

1. Preliminary gut microbiota investigation using PCR-DGGE

1.1 Investigation of gut microbiota of Thai vegetarians and non-vegetarians using PCR-DGGE

The microflora fingerprints for the fecal samples from the 25-65% of denaturant DGGE are shown in Figure 6. Among the 186 DNA bands separated on the polyacrylamide gel, most were within 35-55% of the denaturing gradient. DNA fragments of 10-16 and 13-19 bands were found in each sample from the vegetarian group and non-vegetarian group, respectively. Seventy high intensity bands were cut and identified. Based on a ≥97% identity, only 37 bands were successfully identified (Table 9). The dendrogram of the PCR-DGGE profile shown in Figure 7 was built according to the location of both the unidentified and identified bands and divided into two clusters; A and B. Cluster A consisted of all the non-vegetarians (subjects N1 to N6), while cluster B consisted of all the vegetarians (subjects V1 to V7).

Cluster A was divided into 2 sub-clusters; A1 and A2. *Bacteroides* and *Faecalibacterium prausnitzii* seemed to be the major bacteria detected in sub-cluster A1, which contained subjects N1, N3, N4, N5, and N6, while *Prevotella copri, Roseburia intestinalis* and *F. prausnitzii* were abundant in sub-cluster A2, which only contained subject N2 (Table 10).

The *Bacteroides* in sub-cluster A1 were subsequently identified as four different species; *Bacteroides uniformis, Bacteroides vulgatus, Bacteroides ovatus* and *Bacteroides thetaiotaomicron*. It should be noted that different combinations of *Bacteroides* species were detected in different subjects. While *B. vulgatus* was detected in subjects N1, N3, N5, and N6, *B. uniformis* was found in subjects N3, N5, and N6, yet not in subjects N1 and N4, plus *B. ovatus* and *B. thetaiotaomicron* were only detected in subject N1 (Table 10). With a 97% identity cut-off, no *Bacteroides* species were detected in subject N4. However, *Bacteroides massiliensis* with a low identity of 94% was detected in subject N4. Hence, subject N4 was still grouped in sub-cluster A1. In addition to species of *Bacteriodes*, *F. prausnitzii* was also found in subjects N2, N3, N4, N5, and N6, yet not in

subject N1. *Eubacterium eligenes* and *Megamonas funiformis* with a 97.6 and 100% identity, respectively, were only detected in subject N4.

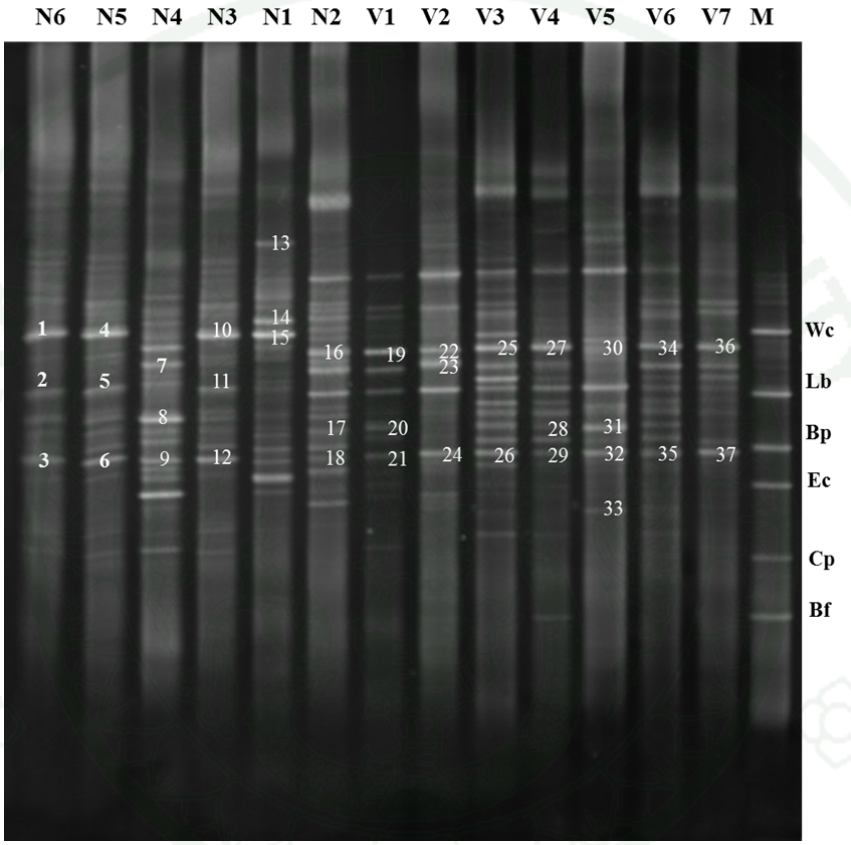

Figure 6 PCR-DGGE profiles of all subjects on 25-65% denaturing gradient gel. Each lane represented one subject. The numbers were marked on the bands that were identified with ≥97% identity by sequencing. Lane M marker, Wc; *Weissella cibraria*, Lb; *Lactobacillus delbruckii* subsp. *Bulgaricus* ATCC11842, Bp; *Bluatia producta* RDB7421, Ec; *Escherichia coli* ATCC25922, Cp; *Clostridium perfringens* ATCC13124 and Bf; *Bifidobacterium pseudocatenulatum* JMC1200.

Table 9 Bacterial species found in 13 subjects with ≥97% identity

Band number	Bacterial species	% identity
1, 4, 10	*Bacteroides uniformis*	97.3-98.0
2, 5, 11, 15	*Bacteroides vulgatus*	97.5-100.0
14	*Bacteroides ovatus*	97.2
13	*Bacteroides thethaiotaomiocron*	97.0
3, 6, 9, 12, 18, 21, 24, 26, 29, 32, 35, 37	*Faecalibacterium prausnitzii*	97.0-98.4
7, 23	*Megamonas funiformis*	100.0
8	*Eubacterium eligens*	97.0
16, 19, 22, 25, 27, 30, 34, 36	*Prevotella copri*	98.0-98.6
17, 20	*Roseburia intestinalis*	100
28, 31	*Eubacterium rectale*	97.6
33	*Clostridium colicanis*	97.3

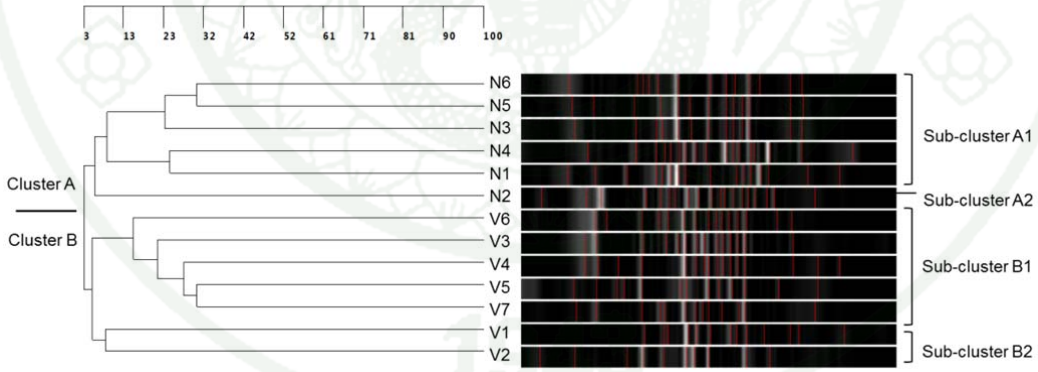

Figure 7 PCR-DGGE profiles of 13 subjects, shown as dendrogram of gut microbiota diversity in vegetarians (V) and non-vegetarians (N), indicating similarity among samples calculated using Dice coefficient and UPGMA algorithm to classify clusters for each community. The scale shows the percentage of similarity. The red lines indicate the position of the DNA bands detected by the SYNGENE Gene Tools software for clustering the PCR-DGGE profile.

Table 10 Bacterial composition found in non-vegetarians and vegetarians determined using PCR-DGGE

Bacterial species / Sample No.	N1	N2	N3	N4	N5	N6	V1	V2	V3	V4	V5	V6	V7
Bacteroides ovatus	•												
Bacteroides thethaiotaomiocron	•												
Bacteroides uniformis			•		•	•							
Bacteroides vulgatus	•		•		•	•							
Clostridium colicanis											•		
Faecalibacterium prausnitzii		•	•	•	•	•	•	•	•	•	•	•	•
Eubacterium eligens				•									
Eubacterium rectale										•	•		
Roseburia intestinalis		•					•						
Megamonas funiformis				•				•					
Prevotella copri		•					•	•	•	•	•	•	•

N; non-vegetarian subject and V; vegetarian subject, •; bacteria present in each subject

It should be noted that the largest microbial portion included unidentified DNA bands with a low identity in the range of 92-94% that belonged to genera *Coprococcus*, *Clostridium*, *Escherichia,* and *Prevotella,* as well as unsuccessfully sequenced DNA bands.

Cluster B included all the vegetarian subjects, V1 to V7. Here, 6 species were detected and classified as *Clostridium colicanis, Eubacterium rectale, F. prausnitzii, M. funiformis, P. copri* and *R. intestinalis*. Two species of *P. copri* and *F. prausnitzii* were found in all the subjects (Table 11). This cluster was also divided into 2 sub-clusters; B1 (subjects V3 to V7) and B2 (subjects V1 and V2), representing the lacto-vegetarians and ovo-lacto-vegetarians, respectively. The subjects in sub-cluster B1 were from the famous Thai vegetarian society in Bangkok called Santi Asoke. Apart from *P. copri* and *F. prausnitzii*, different combinations of bacteria were also detected in each subject. *C. colicanis* was only found in subject V5, while *E. rectale* was found in subjects V4 and V5. For sub-cluster B2, the other species detected were *R. intestinalis* and *M. funiformis,* where *R. intestinalis* was found in subject V1, while *M. funiformis* was observed in subject V2.

It should be noted that the largest microbial portion also included unidentified DNA bands that belonged to the genera *Bacteroides, Clostridium, Escherichia,* and *Prevotella* with low percentage identity values in the range of 90-96%, as well as unsuccessfully sequenced DNA bands.

1.2 Gut microbiota of Thai vegetarians and non-vegetarians by real-time PCR analysis

After the overview of gut microbiota using PCR-DGGE, real-time PCR was consequently used to quantify the specific bacterial groups. All standard curves were created using genomic DNA extracted from the following bacterial cultures: *Prevotella nigrescenes* JCM 12250T, *Bacteroides fragilis* ATCC 25285, *Bifidobacterium bifidum* JCM 1255, *Salmonella Typhimurium* TISTR 292, *Blautia producta* JGD 07421, *Clostridium leptum* DSM 753 and *Lactobacillus casei* subsp. Rhamnosus ATCC 7469 (Appendix E) for *Prevotella, Bacteroides* (based on *Bacteroides fragilis* group)*, Bifidobacterium,* Enterobacteriaceae, *C. coccoides-E. rectale*, *C. leptum,* and *Lactobacillus* groups. The seven bacterial groups found in the subjects were quantified using the log

copy number/g of faeces, as shown in Figure 8. The results showed that *Prevotella*, the *C. coccoides-E. rectale* group, and Enterobacteriaceae were mostly found in the vegetarian subjects. Plus, compared to the non-vegetarians, the numbers of *Prevotella* in the vegetarians (10.4-12.8 log copy number/g) were significantly higher ($p = 0.005$) than those in the non-vegetarians (8.4-9.6 log copy number/g). Among the 6 non-vegetarian subjects, subject N2 showed an exceptionally high number of *Prevotella* with a 12.2 log copy number/g.

In contrast, the non-vegetarian subjects showed higher numbers of *Bacteroides*, the *C. coccoides-E. rectale* group, and *Bifidobacterium*. The numbers of *Bacteroides* in the non-vegetarians (9.7-11.7 log copy number/g) were significantly higher ($p = 0.001$) than those in the vegetarians (9.1-9.9 log copy number/g). As previously mentioned, subject N2 showed higher numbers of *Prevotella* and a high level of *Bacteroides* with a 10.2 log copy number/g. Meanwhile, case N4, showed the highest abundance of Enterobacteriaceae with a 11.9 log copy number/g, yet the lowest level of *Bacteroides* with a 9.7 log copy number/g.

No significant differences ($p > 0.05$) were found between the other bacterial groups studied. The amounts of Enterobacteriaceae, *Lactobacillus*, *Bifidobacterium*, the *C.coccoides-E.rectale* group, and the *C. leptum* group did not differ significantly between the vegetarians and non-vegetarians. The copy numbers of *Lactobacillus* detected in the vegetarian subjects (6.1-8.4 log copy number/g) and non-vegetarian subjects (6.3-7.8 log copy number/g) were the lowest among all the specific bacterial groups determined.

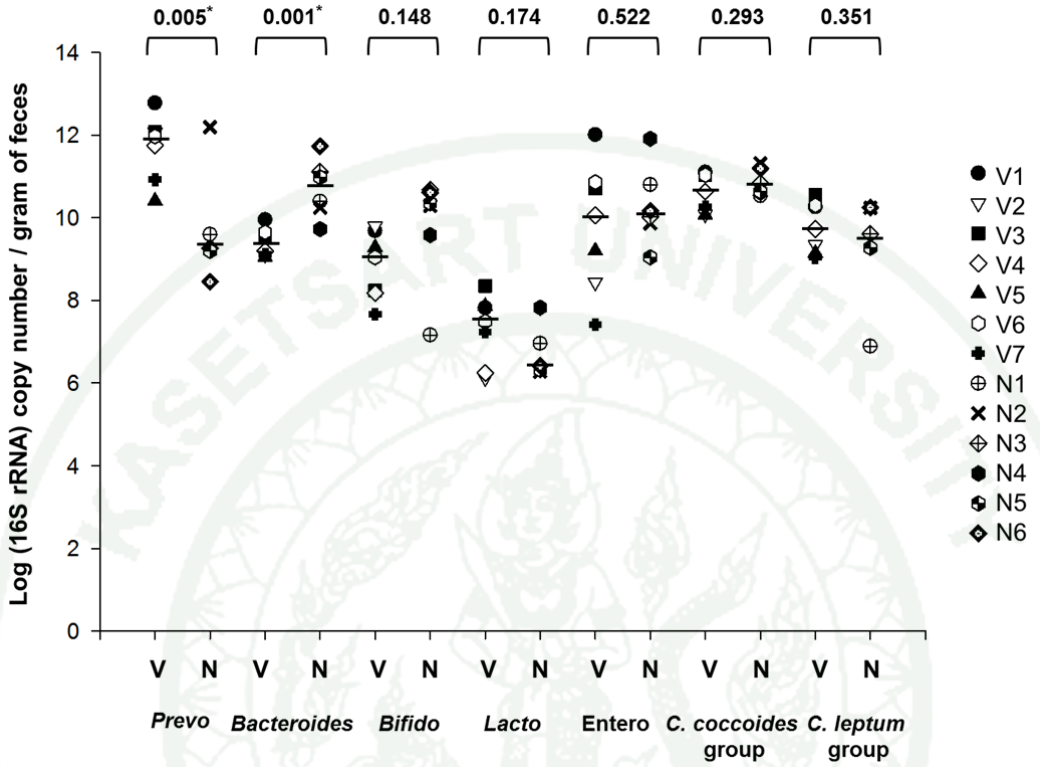

Figure 8 Populations of bacterial groups in all subjects. The copy number for each group was determined using a real-time PCR. The horizontal bars represent the median for the vegetarian (V) and non-vegetarian (N) groups. *Prevo*; *Prevotella* sp., *Bacteroides*; *B. fragilis* group, *Bifido*; *Bifidobacterium* sp., *Lacto*; *Lactobacillus* sp., Entero; Enterobacteriaceae, and *C.coccoides* group; *C. coccoides-E. rectale* group. Significant differences between the two subject groups were determined by a t-test, and the *p*-values are shown at the top of the graph.

2. Gut microbiota analysis of Thai vegetarians and non-vegetarians using real-time PCR and pyrosequencing

Preliminary study of microbiota in 7 Thai vegetarians and 6 non-vegetarians had been analyzed using PCR-DGGE. However, there were still bacterial DNA bands left unidentified. In this part, pyrosequencing, a high throughput method, was used to reveal these unidentified bacterial DNA found in both sample groups. In addition, the number of samples was also increased to 72 healthy Thai subjects: 36 vegetarians and 36 non-vegetarians in this part. The numbers of samples were rearranged differently from preliminary experiment which contained only 13 subjects.

2.1 Characterisation of Thai subjects

The fecal samples were randomly collected from 36 non-vegetarians aged between 41 and 78 years (51.8 ± 8.1 years) and from 36 vegetarians aged between 40 and 61 years (50.9 ± 5.9 years). All the non-vegetarians consumed red meat (only pork), white meat such as fish and chicken,and eggs (9 ± 4 eggs/month), plus only 75% of this group consumed yoghurt and milk (8 cups/month of yoghurt and 12 glasses of milk/month on average). Most of the vegetarians were living and working at Santi-Asoke, exept V1, V2, and V36. All vegetarian volunteers were grouped as ovo-lacto vegetarians (n = 4), lacto-vegetarians (n = 28), ovo-vegetarian (n = 1), and vegans (n = 3). They have been vegetarians for 3 to 35 years (21.2 ± 9.0 years). Among ove-lacto vegetarians and lacto-vegetarians, 16 subjects drank milk 4 glasses of milk/month on average, while 30 subjects consumed yoghurt 7.5 cups/month on average. Only 5 vegetarians consumed egg (1 egg/month on average). The vegetarians and non-vegetarians all consumed Thai fruit on a daily basis. The body mass index (BMI) for the vegetarians was 21.57 ± 2.67 kg/m^2, while that for the non-vegetarians was 24.74 ± 3.52 kg/m^2.

2.2 Investigation of gut microbiota of Thai vegetarians and non-vegetarians using pyrosequencing

From the result of PCR-DGGE and real-time PCR, only genus level was revealed. To gain more insight of these results at the species level, pyrosequencing was applied. To investigate gut microbiota using pyrosequencing, V6-V8 region of 16S rRNA gene was amplified from faeces samples of healthy Thai vegetarians and non-vegetarians. Of the total 273,419 non-chimera reads obtained from both sample groups, 134,800 reads belonged to vegetarians whereas 138,619 reads were from non-vegetarians as shown in Table 11. The average read from vegetarians was 3,744 ± 860 while that from the non-vegetarians was 3,850 ± 851 reads per subject.

Table 11 Summary of gut microbiota analysis using pyrosequencing at phylum to genus level by RDP classifier at ≥0.9 S_ab score

	Identified (number)	Non-chimera	Phylum	%	Class	%	Order	%	Family	%	Genus	%	Species	%
Both group N=72	Taxa	-	9		16		19		41		92		189	
	Sequence (OTU)	893	842	94.29	819	91.71	789	88.35	708	79.28	427	47.82	271	30.35
	Read	273,419	263,210	96.27	262,248	95.91	260,468	95.26	249,702	91.33	202,975	74.24	173,518	63.46
N group N=36	Taxa		8		15		17		39		89		182	
	Sequence (OTU)	794	753	94.84	737	92.82	712	89.67	642	80.86	400	50.38	264	33.25
	Read	138,619	131,934	95.18	131,381	94.78	130,271	93.98	124,603	89.89	97,698	70.48	92,635	66.83
V group N=36	Taxa		9		15		18		39		84		172	
	Sequence (OTU)	762	717	94.09	698	91.60	672	88.19	613	80.45	372	48.82	254	33.33
	Read	134,800	131,276	97.39	130,867	97.08	130,197	96.59	125,099	92.80	105,277	78.10	80,883	60.00

N group; non-vegetarian group and V group; vegetarian group

Based on ≥97% identity, all the reads from both sample groups were dereplicated into 893 OTUs (Figure 9) with 663 OTUs overlapping between both sample groups. Ninety-nine and 131 OTUs were specifically found in vegetarians and non-vegetarians, respectively. Total OTUs found in Thai subjects were identified into 9 phyla, 16 classes, 41 families, 92 genera, and 189 species. This implied diversity of gut microbiota detected.

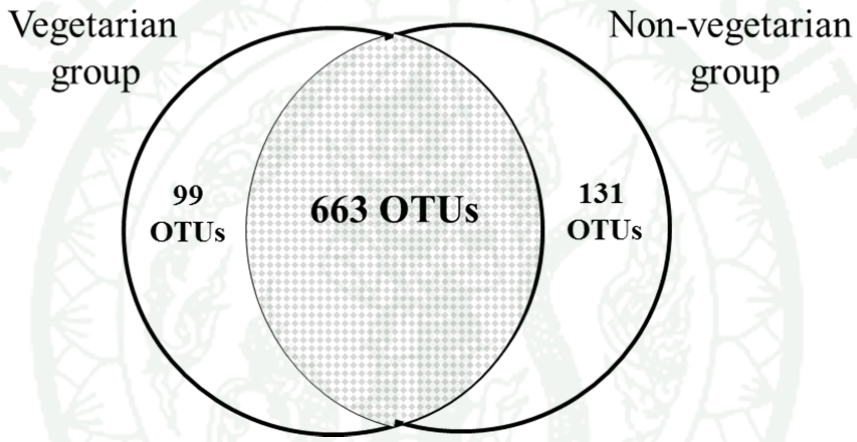

Figure 9 Number of OTUs found in vegetarians and non-vegetarians based on ≥97% identity.

2.2.1 Thai gut microbiota

Gut microbiota of Thai subjects at phylum level were listed in Table 12. From % relative abundance of both groups, more than 85% of detected bacteria belonged to Firmicutes and Bacteroidetes, 10% were accessed to Proteobacteria and Actinobacteria. Less than 1% of gut microbiota were belonged to Verrucomicrobia, Fusobacteria, Cyanobacteria/Chloroplast, Elusimicrobia, and TM7, while 3.73% were unidentified bacteria. Firmicutes and Bacteroidetes were the two largest phyla found in Thai gut.

Table 12 Gut microbiota of Thai subjects at phylum level

Rank	Phylum	N group Relative abundance (%)	V group Relative abundance (%)	Both groups Relative abundance (%)
1	Firmicutes	24.55	23.80	48.35
2	Bacteroidetes	16.17	20.79	36.96
3	Proteobacteria	5.01	2.37	7.38
4	Actinobacteria	1.68	1.01	2.69
5	Verrucomicrobia	0.61	0.034	0.644
6	Fusobacteria	0.22	0.003	0.223
7	Cyanobacteria/Chloroplast	0.004	0.005	0.009
8	Elusimicrobia	0	0.002	0.002
9	TM7	0.001	0.0004	0.001
	Unidentified bacteria	2.44	1.29	3.73
	Total	50.69	49.30	100

Although, both sample groups consumed different diets, the proportions of detected bacteria in both groups were similar at phylum level. The Firmicutes/Bacteroidetes ratio of non-vegetarian group (0.5-212.3) was slightly higher than that of vegetarian group (0.3-13.3). On the contrary, Bacteroidetes/Firmicutes ratio of non-vegetarian group (0.005-1.97) was lower than that of vegetarian group (0.075-3.32) (Figure 10).

Firmicutes/Bacteroidetes ratio of non-vegetarian group was slightly higher than that of vegetarian group. All subjects aged between 40 and 78 years old. It should be noted that there was only one subject aged 78. There was a publication reported that the Firmicutes / Bacteroidetes ratio of adults (25-40 years old) was 10.9 and that of elderly was 0.6 (Mariat *et al*., 2009). This contrast may be due to the age of voluteers in each research. Clemente *et al*. (2012) reviewed that some researches reported that decreasing of Firmicutes/Bacteroidetes ratio was related to obesity and inflammatory bowel disease (IBD), whereas increasing of Bacteroidetes/ Firmicutes ratio was related to type 2 diabetes (Clemente *et al*., 2012). In this study, the Firmicutes/Bacteroidetes ratio of vegetarian group was slightly lower than that of non-vegetarian group and

Bacteroidetes/Firmicutes ratio of vegetarian group was higher than that of non-vegetarian group. So, the vegetarian subjects were likely faced obese, IBD, and type 2 diabetes patients. However, the BMI for the vegetarians (21.57 ± 2.67 kg/m^2) was lower than that for the non-vegetarians (24.74 ± 3.52 kg/m^2). The non-vegetarians have a chance to be overweight subjects. WHO has recommended BMI classification at 18.5-23 kg/m^2 as acceptable risk for Asian populations and reported BMI of overweight of subjects in Thailand (urban) as 25 kg/m^2 (WHO expert consultation, 2004). However, there were no IBD and type 2 diabetes patients in both sample groups (information from interview).

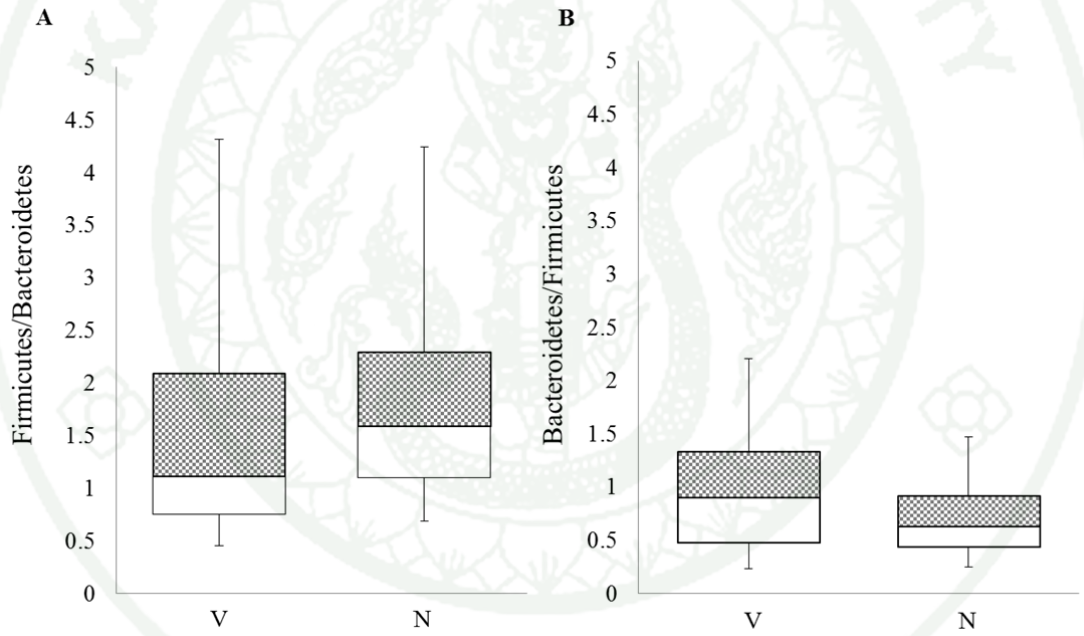

Figure 10 Distribution of Firmicutes to Bacteroidetes (A) and Bacteroidetes to Firmicutes ratios (B) of both sample groups. The white box represented the ratio of 25th to 50th percentile. The checker board box represented the ratio of 50th to 75th percentile. V; vegetarian group and N; non-vegetarian group

The differences of gut microbiota between vegetarians and non-vegetarians appeared at family level. In total 41 families detected in Thai gut microbiota, 11 families were significantly different between both sample groups. They were Bacteroidaceae, Clostridiales_Incertae_Sedis XIII, Flavobacteriaceae, Leuconostocaceae, Pasteurellaceae, Peptococcaceae 1, Porphyromonaceae, Provetellaceae, Rikenellaceae, Sutterllaceae, and Verrucomicrobiaceae. Only the families with >1% relative abundance found in both samples were shown in Figure 11. Among these families, Provetellaceae was high abundance in vegetarians, while Bacteroidaceae was high abundance in non-

vegetarians. These 2 families; Provetellaceae and Bacteroidaceae were significantly different with *p* values of 2.89E-09 and 2.35E-06, respectively. In addition, abundances of Porphyromonaceae, Rikenellaceae and Verrucomicrobiaceae (*p* = 5.77E-05, 3.83E-03 and 2.29E-02, respectively) were significantly different with low % relative abundance. Ruminococcaceae and Lachnospiraceae in both sample groups were found in high amounts but not significantly different between both sample groups (Figure 11).

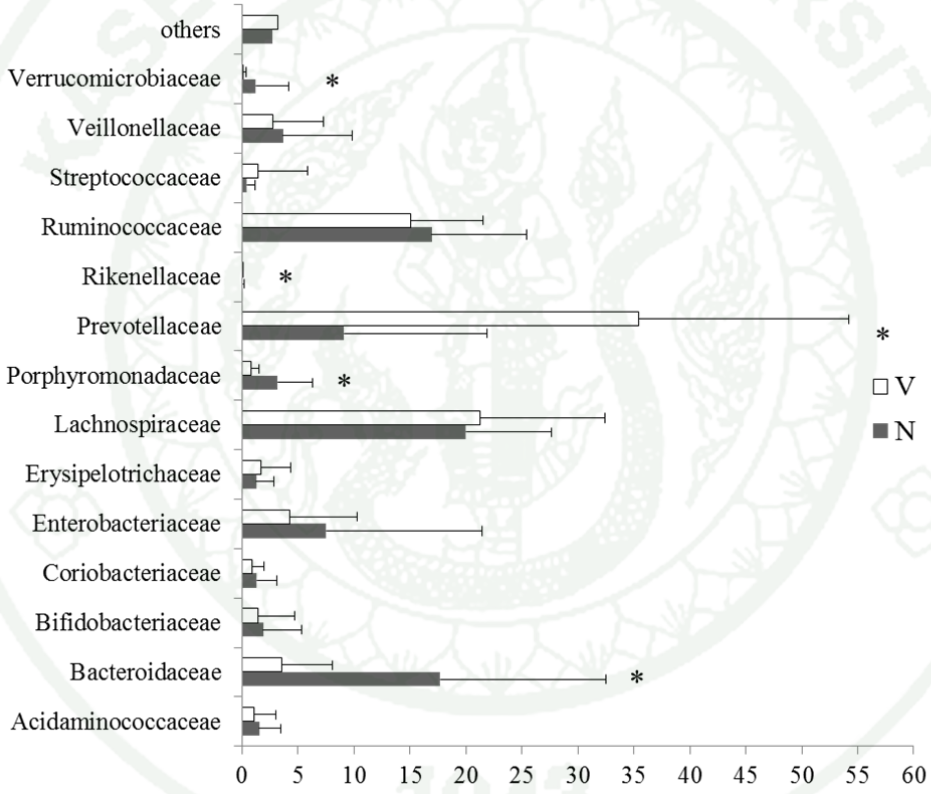

Figure 11 Gut microflora detected in both sample groups at family level. The asterisk indicates families with significantly different. V; vegetarian group and N; non-vegetarian group

Figure 12 showed microflora detected more than 1% relative abundance in both sample groups at genus level. When focusing on genus level, *Prevotella* belonged to Provetellaceae and *Bacteroides* belonged to Bacteroidaceae were different abundances in both sample groups. *Prevotella* was high abundance in vegetarians, while *Bacteroides* was high abundance in non-vegetarians. This result was agreed with the result from PCR-DGGE. The other genera; *Akkermansia* belonged to the family Verrucomicrobiaceae, *Alistipes* belonged to the family Rikenellaceae, bacterial genus closed

to *Klebsiella* belonged to the family Enterobacteriaceae, and *Parabacteroides* belonged to the family Porphyromonaceae were significantly different but they were low abundances. The concentration of the genus *Alistipes* in non-vegetarians (1.49 % relative abundance on average) was higher than that of vegetarians (0.14 % relative abundance on average), while that of the genus *Akkermansia* in non-vegetarians (1.24 % relative abundance on average) was higher than that of vegetarians (0.068 % relative abundance on average). However, *Faecalibacterium* belonged to the family Ruminococcaceae and *Lachnospiracea_incertae_ sedis* belonged to the family Lachnospiraceae and were found high amount but not yet significantly different between vegetarians and non-vegetarians (Figure 12). These results were complementary with the results at family level as mentioned above, except the family Enterobacteriaceae.

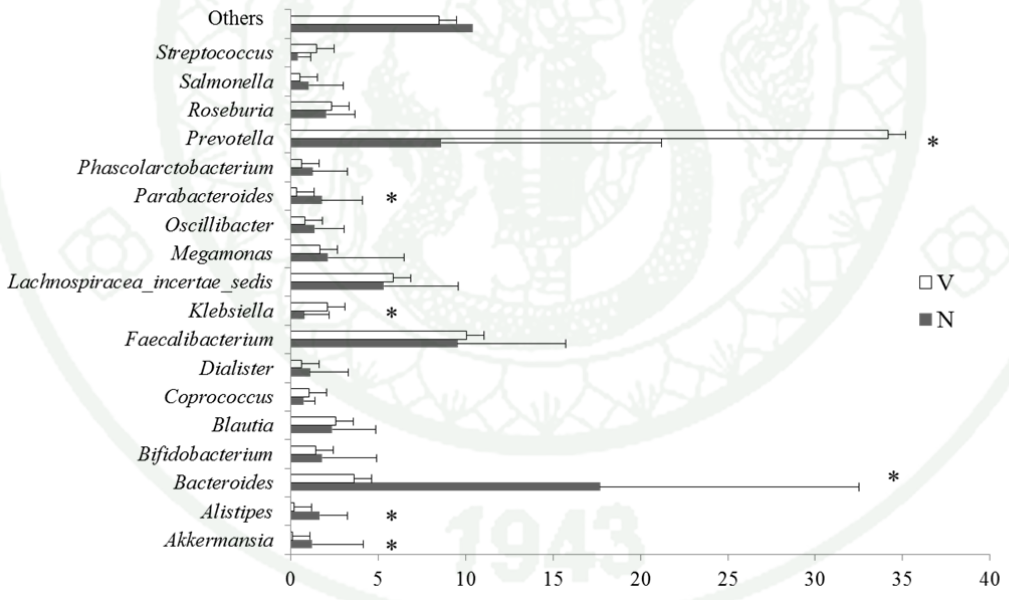

Figure 12 Gut microflora detected in both sample groups at genus level. The asterisk indicates genera with significantly different. V; vegetarian group and N; non-vegetarian group.

When datasets of all bacteria analyzed by pyrosequencing at species level were subjected to principal component analysis (PCA), the separation of vegetarian and non-vegetarian samples following to their specific characteristic microbiota was illustrated (Figure 13). Considering to axis 1 of PCA plot, *P. copri* was the key species of vegetarians whereas *Bacteroides vulgatus* was the key species for non-vegetarians. Apart from the key species *B. vulgatus*, bacterial species closed to *Escherichia hermanii* and *E. coli* may be used to indicate the non-vegetarians. However, *F. prausnitzii* were found in both sample groups without significantly different concentration implying that this bacterium could not be used to differentiate non-vegetarians and vegetarians.

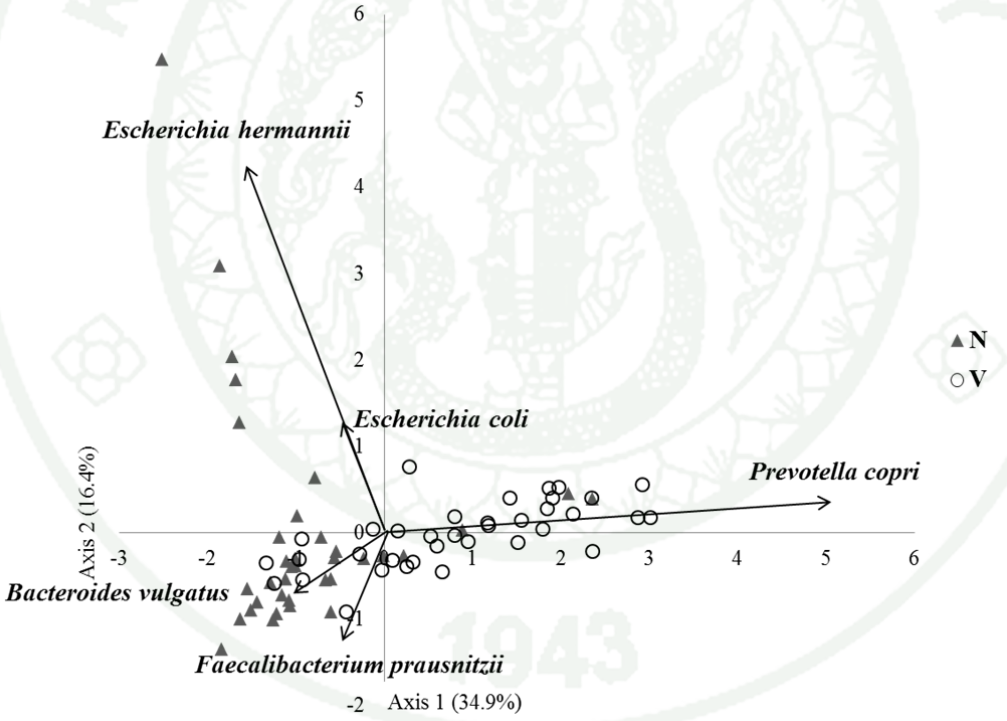

Figure 13 Species level bacterial compositions analysis using PCA plot.

2.2.2 Core gut microbiota of Thai subjects

In this study, core microbiota was defined as bacterial species which are found not less than 80% of total subjects. As a consequence, top 20 bacterial species detected for more than 80% of Thai subjects were listed in Table 13. They were belonging to Firmicutes (19.62% relative abundance), Bacteroidetes (16.28% relative abundance), Proteobacteria (6.81% relative abundance), and Actinobacteria (0.79% relative abundance) phyla. *P. copri, Bacteroides uniformis, B. vulgatus, Parabacteroides distasonis,* were in Bacteroidetes phylum. *F. prausnitzii, E. rectale, E. eligens, Roseburia inulinivorans, Blautia wexlerae, Streptococus thermophilus, Eubacterium hadrum, Roseburia faecis, Ruminococcus torques,* and *Ruminococcus obeum* were in Firmicutes phylum. Bacterial species closed to *Escherichia hermanii*, *Klebsiella pneumoniae, E. coli,* and *Gemmiger formicilis* were in Proteobacteria phylum. *Collinsella aerofaciens* was in Actinobacteria. Among these core species, the largest bacterial group in Firmicutes was *C. coccoides-E. rectale* group.

In addition to the top 20 bacteria species shown, including *Clostridium clostridioforme*, *C. nexile*, *Dorae formicigenerans*, and *D. Longicatena*, which also present in *C. coccoides-E. rectale* group were also found in ≥80% of total subjects.

Table 13 Major microorganisms found in more than 80% of all Thai subjects

Rank	OTU No.	Species	Relative abundance (%)	Carriers (subjects)
1	2, 4, 10, 12, 17, 204, 264, 390, 416, 599, 607, 608, 611, 677, 678, 844	*Faecalibacterium prausnitzii*	10.50	72
2	68, 175, 226, 419, 467, 613, 693, 799	*Prevotella copri*	10.35	62
3	3, 757	*Eubacterium rectale*	2.99	66
4	5, 859	*Bacteroides vulgatus*	2.88	65
5	1	*Escherichia hermannii*	2.71	66
6	9, 135, 730	*Eubacterium eligens*	1.51	66
7	8, 629	*Klebsiella pneumoniae*	1.48	67
8	16, 40, 537, 838	*Roseburia inulinivorans*	1.47	70
9	11, 466, 716	*Bacteroides uniformis*	1.42	64
10	301, 509, 529, 615, 748, 810	*Escherichia coli*	1.09	65
11	360, 406	*Gemmiger formicilis*	1.08	72
12	20, 825	*Blautia wexlerae*	0.93	69
13	23	*Streptococus thermophilus*	0.87	60
14	18, 158, 869, 873	*Collinsella aerofaciens*	0.79	66
15	32, 207	*Parabacteroides distasonis*	0.70	65
16	26, 614	*Eubacterium hadrum*	0.69	62
17	58, 566, 625, 724, 819	*Roseburia faecis*	0.59	60
18	44, 162, 556, 581, 775, 823	*Ruminococcus torques*	0.53	65
19	75, 89, 464	*Ruminococcus obeum*	0.47	70
20	51	*Raoultella ornithinolytica*	0.45	59

Top 20 bacterial species detected in ≥80% of total of non-vegetarians and vegetarians were listed as shown in Table 14 and 15, respectively. Only 2 species found in all subjects were *F. prausnitzii* with 10.63 and 10.36% relative abundance for non-vegetarians and vegetarian groups, respectively and *G. formicilis* with 1.33 and 0.84% relative abundance for non-vegetarians and vegetarian groups, respectively. *F. Prausnitzii* belonged to the phylum Firmicutes and *G. formicilis* belonged to the phylum Actinobacteria. Moreover, *Parabacteroides distasonis* belonged to the phylum Bacteroidetes found all non-vegetarians (1.22% relative abundance). In addition, *Roseburia inulinivorans* and *Ruminococcus obeum* belonged to *C. coocoides-E. rectale* group in the phylum Firmicutes were detected in most subjects (35 vegetarians and 35 non-vegetarians). The high abundances of core species in Thai subjects were *F. prausnitzii, P. copri, E. rectale,* and *B. vulgatus*.

These results showed that core gut microbiota of Thai people were *F. prausnitzii,* various species of *Bacteroides*, *P. copri* and short chain fatty acid (SCFA) producing bacteria including *C. coccoides-E. rectale* and *C. leptum* groups. From the results, it was clearly suggested that *Bacteroides* and *Prevotella* enterotypes were attributions for the core gut microbiota of non-vegetarians and vegetarians, respectively.

Table 14 Major Microorganisms found in more than 80% of Thai non-vegetarians

Rank	OTU No.	Species	Relative abundance (%)	Carriers (subjects)
1	2, 4, 10, 12, 17, 204, 264, 390, 416, 599, 607, 608, 611, 677, 678, 844	*Faecalibacterium prausnitzii*	10.63	36
2	1	*Escherichia hermannii*	4.70	35
3	5, 859	*Bacteroides vulgatus*	4.50	33
4	68, 175, 226, 419, 467, 613, 693, 799	*Prevotella copri*	3.80	28
5	6, 609, 785	*Bacteroides dorei*	3.03	28
6	11, 466, 716	*Bacteroides uniformis*	2.45	33
7	3, 757	*Eubacterium rectale*	2.14	33
8	9, 135, 730	*Eubacterium eligens*	1.82	32
9	301, 509, 529, 615, 748, 810	*Escherichia coli*	1.53	34
10	16, 40, 537, 838	*Roseburia inulinivorans*	1.36	35
11	360, 406	*Gemmiger formicilis*	1.33	36
12	32, 207	*Parabacteroides distasonis*	1.22	36
13	27	*Alistipes putredinis*	0.96	29
14	18, 158, 869, 873	*Collinsella aerofaciens*	0.95	34
15	20, 825	*Blautia wexlerae*	0.81	34
16	8, 629	*Klebsiella pneumoniae*	0.79	34
17	26, 614	*Eubacterium hadrum*	0.78	33
18	58, 566, 625, 724, 819	*Roseburia faecis*	0.65	31
19	59, 132	*Clostridium nexile*	0.54	32
20	44, 162, 556, 581, 775, 823	*Ruminococcus torques*	0.53	34

Table 15 Major Microorganisms found in more than 80% of Thai vegetarians

Rank	OTU No.	Species	Relative abundance (%)	Carriers (subjects)
1	68, 175, 226, 419, 467, 613, 693, 799	*Prevotella copri*	16.90	34
2	2, 4, 10, 12, 17, 204, 264, 390, 416, 599, 607, 608, 611, 677, 678, 844	*Faecalibacterium prausnitzii*	10.36	36
3	3, 757	*Eubacterium rectale*	3.84	33
4	8, 629	*Klebsiella pneumoniae*	2.17	33
5	16, 40, 537, 838	*Roseburia inulinivorans*	1.59	35
6	23	*Streptococus thermophilus*	1.42	32
7	5, 859	*Bacteroides vulgatus*	1.27	32
8	9, 135, 730	*Eubacterium eligens*	1.20	34
9	20, 825	*Blautia wexlerae*	1.04	35
10	360, 406	*Gemmiger formicilis*	0.84	36
11	1	*Escherichia hermannii*	0.72	31
12	301, 509, 529, 615, 748, 810	*Escherichia coli*	0.65	31
13	18, 158, 869, 873	*Collinsella aerofaciens*	0.64	32
14		*Raoultella ornithinolytica*	0.62	31
15	26, 614	*Eubacterium hadrum*	0.59	29
16	75, 89, 464	*Ruminococcus obeum*	0.57	35
17	58, 566, 625, 724, 819	*Roseburia faecis*	0.54	29
18	44, 162, 556, 581, 775, 823	*Ruminococcus torques*	0.53	31
19	11, 466, 716	*Bacteroides uniformis*	0.40	31
20	45, 894	*Dorea longicatena*	0.36	34

2.2.3 An opportunistic pathogen or pathogen in Thai subjects

Although, bacterial species closed to *Escherichia coli, E. hermannii,* and *Klebsella pneumoniae* were normal microflora in human GI tract, some strains were opportunistic pathogens. It was noted that bacterial species closed to *E. coli, E. hermannii,* and *Klebsella pneumoniae* were found in 65, 66, and 67 subjects, respectively (Table 16). The bacterial species closed to *E. coli* were 1.53% relative abundance in non-vegetarian group and 0.65% relative abundance in vegetarian group while bacterial species closed to *E. hermannii* were 4.70% relative abundance in non-vegetarian group and 0.72% relative abundance in vegetarian group. For the bacterial species closed to *K. pneumoniae,* were 0.79% and 2.17% relative abundance in non-vegetarian group and in vegetarian group, respectively (Table 14 and 15). In addition, *Clostridium perfringens*, an enteric pathogen caused diarrhea, was found in 12 non-vegetarians while it was detected in only 2 vegetarians in very low concentration. On the other hand, in prevalence of *Pantoea agglomerans* (a plant pathogen) in vegetarian samples was higher than that of non-vegetarian samples. Moreover, *Bilophila wadsworthia* were higher concentration and prevalence in non-vegetarians, while *Fusobacterium nucleatum*, associated with human colorectal carcinoma was found in only 3 vegetarians with very low detection.

Table 16 The opportunistic pathogens found in Thai gut

OTU No	Opportunistic pathogens / associated disease bacteria	Non-vegetarians		Vegetarians	
		Relative abundance (%)	carrier (subjects)	Relative abundance (%)	carrier (subjects)
216	*Clostridium perfringens*	0.037	12	0.007	2
301, 509, 529, 615, 748, 810	*Esherichia coli*	1.526	34	0.652	31
1	*Esherichia hermannii*	4.703	35	0.716	31
8, 629	*Klebsella pneumoniae*	0.793	34	2.170	33
828	*Pantoea agglomerans*	0.053	19	0.121	31
155	*Bilophila wadsworthia*	0.166	31	0.014	11
773	*Fusobacterium nucleatum*	0	0	0.002	3

2.3 Quantification of specific group of gut microbiota of Thai vegetarians and non-vegetarians using real-time PCR

The quantification of 8 specific groups: *Prevotella, Bacteroides*, *Bifidobacterium, Lactobacillus,* Enterobacteriaceae, the *C. coccoides-E. rectale* group, the *C. leptum* group, and the *F. prausnitzii-S. variabile* like bacteria in each subject were carried out. In previous reports, these specific groups usually found in human gut microbiota and benefit for human health, so, they were chosen to determine their quantity in each sample. The amounts of all bacterial specific groups detected in both vegetarian and non-vegetarian groups using real-time PCR were shown in Table 17 to 24. They were summarised in Table 25 and Figure 14.

Table 17 Determination of *Prevotella* in each sample

Sample code	Log_{10} copy number / gram of faeces	Sample code	Log_{10} copy number / gram of faeces	Sample code	Log_{10} copy number / gram of faeces	Sample code	Log_{10} copy number / gram of faeces
N1	12.19	N19	10.48	V1	12.78	V19	11.82
N2	9.27	N20	10.70	V2	12.08	V20	12.06
N3	9.27	N21	5.17	V3	11.75	V21	11.76
N4	9.20	N22	9.90	V4	12.08	V22	11.76
N5	8.45	N23	7.16	V5	12.18	V23	11.88
N6	9.56	N24	11.24	V6	10.41	V24	12.10
N7	9.18	N25	11.09	V7	11.97	V25	10.39
N8	9.25	N26	10.49	V8	12.06	V26	12.40
N9	10.45	N27	7.12	V9	11.89	V27	9.75
N10	9.53	N28	11.41	V10	10.92	V28	11.46
N11	9.60	N29	7.54	V11	10.11	V29	12.07
N12	11.42	N30	10.75	V12	11.08	V30	11.76
N13	11.29	N31	11.35	V13	11.62	V31	10.34
N14	11.56	N32	10.89	V14	11.73	V32	11.15
N15	9.17	N33	7.10	V15	7.29	V33	11.88
N16	9.78	N34	7.73	V16	11.59	V34	12.20
N17	10.54	N35	10.42	V17	11.73	V35	11.39
N18	10.95	N36	7.31	V18	9.48	V36	11.62

Table 18 Determination of *Bacteroides fragilis* group in each sample

Sample code	Log_{10} copy number / gram of faeces	Sample code	Log_{10} copy number / gram of faeces	Sample code	Log_{10} copy number / gram of faeces	Sample code	Log_{10} copy number / gram of faeces
N1	10.25	N19	11.69	V1	9.95	V19	11.33
N2	11.12	N20	9.80	V2	9.11	V20	10.89
N3	9.72	N21	10.42	V3	9.19	V21	9.20
N4	10.98	N22	9.40	V4	9.57	V22	10.45
N5	11.73	N23	11.15	V5	10.82	V23	9.92
N6	11.25	N24	11.16	V6	9.05	V24	9.72
N7	1.86	N25	11.13	V7	9.65	V25	10.25
N8	11.93	N26	11.30	V8	11.50	V26	10.30
N9	11.4	N27	11.04	V9	10.66	V27	10.07
N10	11.72	N28	11.12	V10	9.11	V28	9.69
N11	12.08	N29	11.61	V11	10.98	V29	10.52
N12	11.00	N30	11.39	V12	11.00	V30	8.70
N13	11.05	N31	11.00	V13	1.045	V31	8.97
N14	10.23	N32	10.84	V14	10.92	V32	9.20
N15	11.28	N33	11.12	V15	9.66	V33	9.98
N16	11.49	N34	9.84	V16	10.66	V34	10.69
N17	11.38	N35	10.32	V17	10.32	V35	9.93
N18	11.13	N36	10.49	V18	8.99	V36	9.12

Table 19 Determination of *Bifidobacterium* in each sample

Sample code	Log_{10} copy number / gram of faeces	Sample code	Log_{10} copy number / gram of faeces	Sample code	Log_{10} copy number / gram of faeces	Sample code	Log_{10} copy number / gram of faeces
N1	10.29	N19	9.96	V1	9.68	V19	9.02
N2	10.68	N20	8.96	V2	9.79	V20	10.05
N3	9.58	N21	7.74	V3	8.18	V21	10.63
N4	10.36	N22	8.91	V4	8.24	V22	9.82
N5	10.61	N23	10.30	V5	10.15	V23	10.20
N6	8.65	N24	9.12	V6	9.28	V24	10.85
N7	10.66	N25	10.31	V7	9.04	V25	8.76
N8	9.20	N26	10.50	V8	10.77	V26	10.14
N9	10.51	N27	10.44	V9	10.19	V27	10.61
N10	9.36	N28	10.77	V10	7.67	V28	10.42
N11	10.02	N29	10.14	V11	9.79	V29	7.14
N12	9.39	N30	9.94	V12	9.89	V30	11.11
N13	7.92	N31	10.55	V13	9.16	V31	9.99
N14	10.57	N32	7.60	V14	9.89	V32	9.42
N15	7.91	N33	10.17	V15	7.64	V33	10.44
N16	10.16	N34	10.89	V16	9.51	V34	10.55
N17	11.24	N35	10.48	V17	9.38	V35	8.82
N18	9.98	N36	9.62	V18	10.52	V36	8.17

Table 20 Determination of *Lactobacillus* in each sample

Sample code	Log_{10} copy number / gram of faeces	Sample code	Log_{10} copy number / gram of faeces	Sample code	Log_{10} copy number / gram of faeces	Sample code	Log_{10} copy number / gram of faeces
N1	6.28	N19	7.37	V1	7.82	V19	6.49
N2	6.42	N20	3.96	V2	6.12	V20	8.51
N3	7.83	N21	7.04	V3	6.24	V21	7.36
N4	6.32	N22	7.39	V4	8.35	V22	8.14
N5	6.42	N23	9.01	V5	8.28	V23	6.83
N6	8.01	N24	8.96	V6	7.86	V24	7.16
N7	7.70	N25	7.17	V7	7.49	V25	8.63
N8	7.36	N26	8.07	V8	7.76	V26	8.44
N9	8.22	N27	7.10	V9	6.57	V27	8.34
N10	7.51	N28	7.86	V10	7.23	V28	8.84
N11	7.34	N29	7.74	V11	6.05	V29	8.96
N12	7.00	N30	6.57	V12	7.11	V30	8.09
N13	6.84	N31	7.68	V13	6.38	V31	8.73
N14	8.32	N32	6.69	V14	7.69	V32	8.21
N15	7.35	N33	8.04	V15	6.05	V33	7.54
N16	6.93	N34	7.85	V16	7.40	V34	7.75
N17	7.12	N35	8.19	V17	9.04	V35	7.79
N18	6.23	N36	7.70	V18	8.63	V36	7.86

Table 21 Determination of Enterobacteriaceae in each sample

Sample code	Log_{10} copy number / gram of faeces	Sample code	Log_{10} copy number / gram of faeces	Sample code	Log_{10} copy number / gram of faeces	Sample code	Log_{10} copy number / gram of faeces
N1	9.87	N19	111.57	V1	12.01	V19	8.65
N2	10.02	N20	11.12	V2	8.44	V20	10.77
N3	11.91	N21	10.36	V3	10.06	V21	11.08
N4	9.04	N22	12.23	V4	10.70	V22	10.78
N5	10.16	N23	12.18	V5	11.50	V23	9.56
N6	9.00	N24	11.48	V6	9.20	V24	11.67
N7	11.54	N25	11.14	V7	10.86	V25	11.15
N8	11.22	N26	11.53	V8	10.34	V26	11.05
N9	11.64	N27	11.00	V9	8.80	V27	10.50
N10	10.90	N28	11.84	V10	7.41	V28	11.18
N11	10.01	N29	11.73	V11	110.77	V29	10.25
N12	11.54	N30	11.34	V12	11.62	V30	10.52
N13	11.15	N31	12.01	V13	1054	V31	9.74
N14	10.93	N32	10.62	V14	11.16	V32	9.46
N15	11.16	N33	11.94	V15	8.98	V33	10.83
N16	9.22	N34	9.97	V16	11.41	V34	11.41
N17	12.23	N35	10.19	V17	12.01	V35	10.35
N18	11.90	N36	12.25	V18	10.35	V36	10.94

Table 22 Determination of *Clostridium coccoides-Eubacterium rectale* group in each sample

Sample code	Log_{10} copy number / gram of faeces	Sample code	Log_{10} copy number / gram of faeces	Sample code	Log_{10} copy number / gram of faeces	Sample code	Log_{10} copy number / gram of faeces
N1	11.32	N19	11.32	V1	11.10	V19	11.05
N2	10.84	N20	11.10	V2	10.09	V20	11.11
N3	10.61	N21	10.01	V3	10.64	V21	11.20
N4	10.63	N22	9.72	V4	11.04	V22	10.69
N5	11.19	N23	10.66	V5	11.45	V23	11.43
N6	11.14	N24	11.14	V6	10.07	V24	11.42
N7	10.98	N25	10.97	V7	11.03	V25	10.80
N8	11.43	N26	10.75	V8	11.51	V26	11.50
N9	11.47	N27	11.11	V9	10.87	V27	10.74
N10	11.08	N28	11.25	V10	10.26	V28	11.46
N11	10.80	N29	11.28	V11	10.81	V29	11.43
N12	11.33	N30	11.17	V12	1097	V30	10.09
N13	10.94	N31	11.63	V13	10.72	V31	10.80
N14	11.05	N32	11.15	V14	11.27	V32	10.73
N15	11.36	N33	11.28	V15	10.00	V33	11.20
N16	11.19	N34	10.84	V16	11.11	V34	11.46
N17	11.05	N35	10.82	V17	11.37	V35	10.96
N18	11.02	N36	10.85	V18	10.30	V36	10.98

Table 23 Determination of *Clostridium leptum* group in each sample

Sample code	Log_{10} copy number / gram of faeces	Sample code	Log_{10} copy number / gram of faeces	Sample code	Log_{10} copy number / gram of faeces	Sample code	Log_{10} copy number / gram of faeces
N1	10.24	N19	10.66	V1	10.26	V19	9.69
N2	9.61	N20	10.17	V2	9.35	V20	10.15
N3	9.27	N21	9.89	V3	9.73	V21	10.32
N4	9.27	N22	9.26	V4	10.56	V22	10.14
N5	10.25	N23	10.30	V5	10.83	V23	10.49
N6	10.28	N24	10.79	V6	9.15	V24	10.50
N7	10.18	N25	10.56	V7	10.30	V25	9.63
N8	10.68	N26	10.64	V8	9.40	V26	10.87
N9	10.52	N27	10.86	V9	10.14	V27	9.55
N10	10.73	N28	10.91	V10	9.04	V28	10.58
N11	10.21	N29	10.74	V11	9.82	V29	10.38
N12	10.57	N30	11.04	V12	10.35	V30	9.08
N13	10.63	N31	10.96	V13	9.94	V31	9.48
N14	10.68	N32	10.66	V14	10.41	V32	9.81
N15	10.53	N33	10.85	V15	8.55	V33	10.75
N16	10.47	N34	10.04	V16	9.81	V34	10.28
N17	10.21	N35	10.03	V17	9.39	V35	9.83
N18	10.21	N36	10.01	V18	8.99	V36	9.49

Table 24 Determination of *Faecalibacterium prausnitzii-Subdoligranulum variabile* like bacteria in each sample

Sample code	Log_{10} copy number / gram of faeces	Sample code	Log_{10} copy number / gram of faeces	Sample code	Log_{10} copy number / gram of faeces	Sample code	Log_{10} copy number / gram of faeces
N1	9.47	N19	10.02	V1	9.79	V19	9.58
N2	9.78	N20	9.36	V2	9.50	V20	9.84
N3	9.22	N21	9.83	V3	9.44	V21	9.84
N4	9.53	N22	9.29	V4	9.99	V22	9.80
N5	10.00	N23	9.78	V5	10.15	V23	9.92
N6	10.10	N24	10.25	V6	9.44	V24	10.06
N7	9.56	N25	10.11	V7	9.67	V25	9.58
N8	10.29	N26	10.01	V8	10.09	V26	10.16
N9	10.21	N27	10.33	V9	9.76	V27	9.36
N10	10.21	N28	10.41	V10	9.61	V28	10.31
N11	9.77	N29	10.14	V11	9.38	V29	9.95
N12	10.00	N30	10.33	V12	10.08	V30	9.08
N13	10.26	N31	10.29	V13	9.46	V31	9.38
N14	10.06	N32	9.82	V14	9.82	V32	9.81
N15	10.06	N33	10.32	V15	6.72	V33	10.11
N16	10.37	N34	9.51	V16	9.74	V34	9.63
N17	9.93	N35	9.54	V17	10.04	V35	9.58
N18	9.79	N36	9.41	V18	9.04	V36	9.20

Table 25 Summary of specific group determination using real-time PCR

Specific genus / group	Vegetarian		Non-vegetarian		*p* value
	median	S.D.	median	S.D.	
Prevotella	11.76	1.03	9.84	1.61	1.2E-06
Bacteroides fragilis group	9.97	0.77	11.13	0.65	9E-08
Bifidobacterium	9.81	0.99	10.15	0.94	0.2996
Enterobacteriaceae	10.74	1.06	11.23	0.93	0.0114
Clostridium coccoides group	11.01	0.45	11.09	0.37	0.4274
Clostridium leptum group	9.88	0.58	10.50	0.47	0.0007
Lactobacillus	7.77	0.88	7.36	0.91	0.1136
Faecalibacterium prausnitzii-Subdoligranulum variabile like bacteria	9.75	0.59	10.00	0.34	0.0137

From the results, the amount of *Prevotella*, *Bacteroides,* Enterobacteriaceae, the *C. leptum,* and the *F. prausnitzii-S. variabile* like bacteria group were significantly different between both groups at *p* value 1.2E-06, 9E-08, 0.0114, 0.0007, and 0.0137, respectively. *Prevotella* was the largest group in the vegetarian whereas Enterobacteriaceae was detected most in the non-vegetarian. Though the amount of *C.leptum* in both vegetarians and non-vegetarians showed different values, the major bacteria detected in *C. leptum* of both sample groups were *F. prausnitzii-S. variabile* like bacteria group (Table 25).

No significant differences ($p > 0.05$) were found between the other bacterial groups studied. The copy number of *Lactobacillus* group detected in both vegetarian subjects (6.0-9.0 log copy number/g of faeces) and non-vegetarian volunteers (4.0-9.0 log copy number/g of faeces) were the lowest among any of specific bacterial groups determined, while those of *Bifidobacterium* found in vegetarian subjects were between 7.1 and 11.1 log copy number/g of faeces and non-vegetarian volunteers were 7.6 and 11.2 log copy number/g of faeces. The amount of *C. coccoides-E. rectale* group in vegetarian and non-vegetarian groups varied in narrow range of 10.1-11.5 and 9.7-11.6 log copy number/g of faeces, respectively.

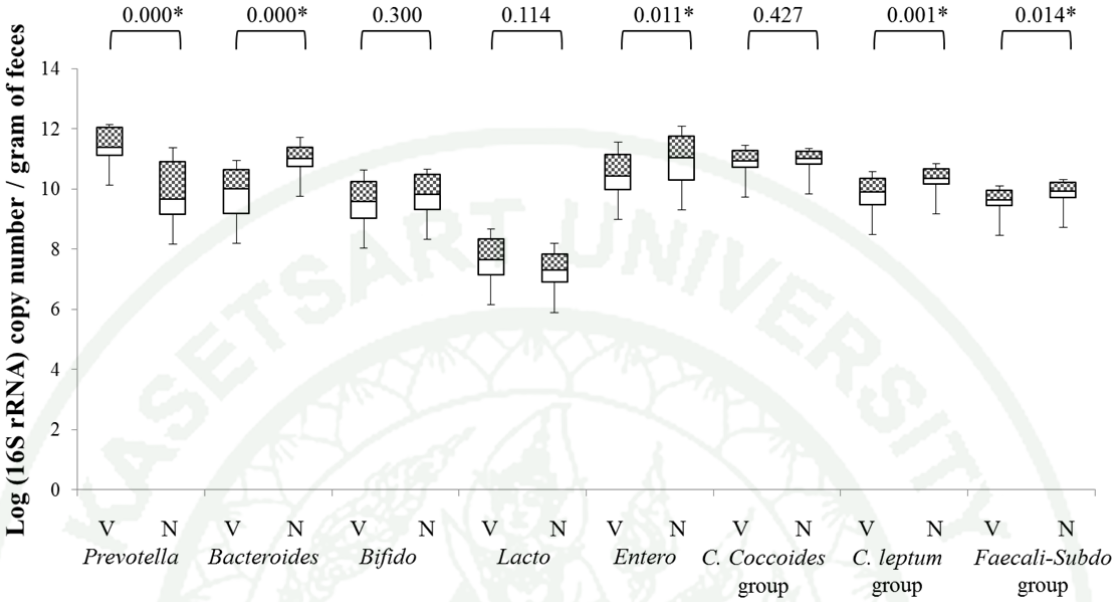

Figure 14 The copy number for each bacterial group were determined using a real-time PCR. The horizontal bars represent the median for the vegetarian (V) and non-vegetarian (N) groups. *Bifido*; *Bifidobacterium* sp., *Lacto*; *Lactobacillus* sp., Entero; Enterobacteriaceae, *C.coccoides* group; *C. coccoides-E. rectale* group and *Faecali-Subdo* group; *Faecalibacterium prusnitzii-Subdoligranulum variabile* like bacteria group. Significant differences between the two subject groups were determined by a t-test, and the *p*-values are shown at the top of the graph. The white box represented the ratio of 25th to 50th percentile. The checker board box represented the ratio of 50th to 75th percentile.

When the amount of all specific bacterial group were analysed the correlation between each specific bacterial group, the amount of Enterobacteriaceae were positively related to those of *C. leptum* and *F. prausnitzii-S. variabile* like bacteria group when analysed by real-time PCR (Figure 15).

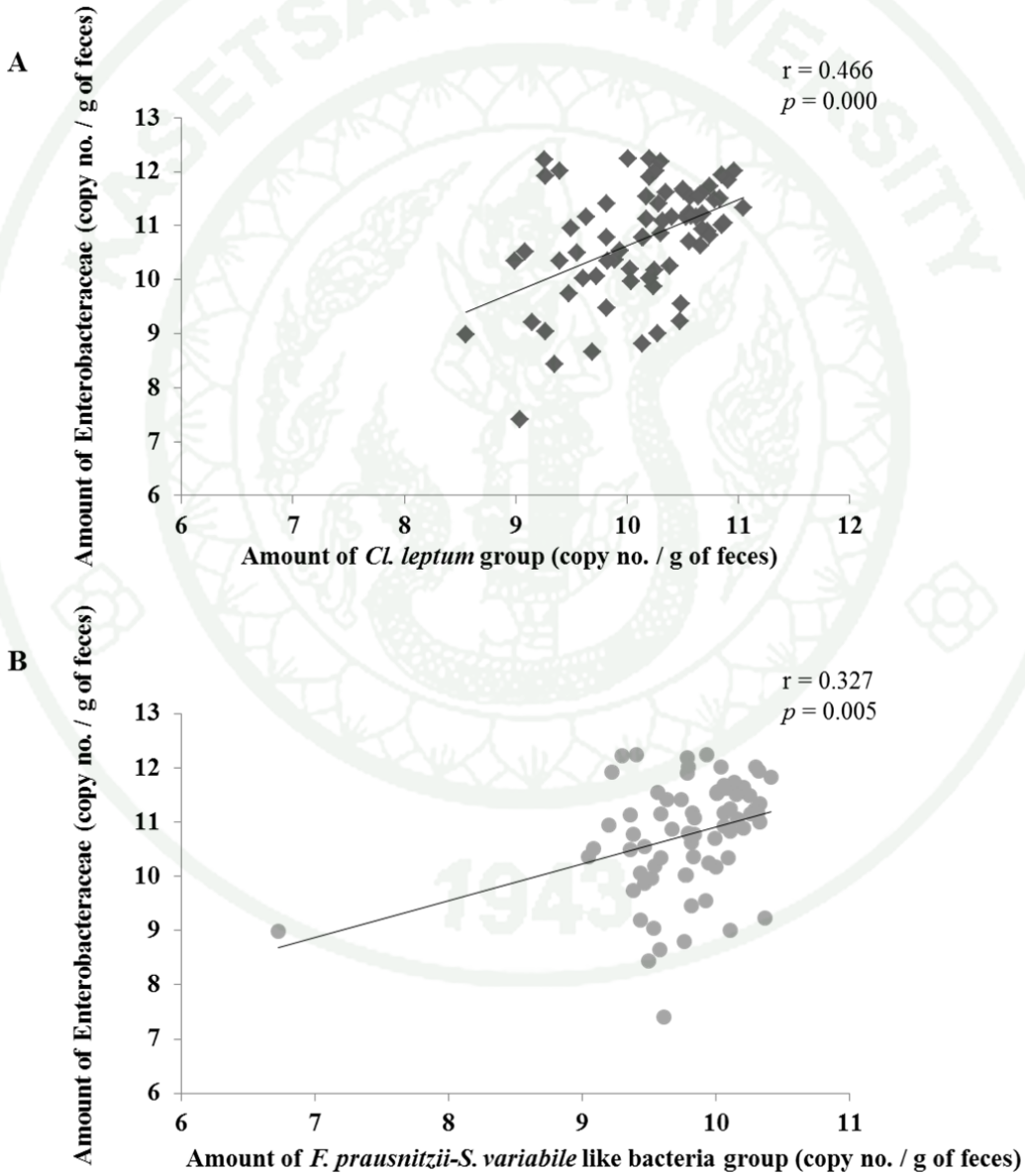

Figure 15 Correlations between amount of Enterobacteriaceae and *C. leptum* group (A) and between amount of Enterobacteriaceae and *F. prausnitzii-S. variabile* like bacteria group (B).

2.4 Comparative investigation of gut microbiota of Thai vegetarians and non-vegetarians using pyrosequencing and real-time PCR

From Table 25 and Figure 14 which showed the amounts of specific bacterial groups detected in both sample groups using real-time PCR, *Prevotella, B. fragilis* group, Enterobacteriaceae, *C. leptum* group, and *F. prausnitzii-S. variabile* like bacteria group were significantly different between both vegetarian and non-vegetarian groups. Meanwhile, pyrosequencing was used for analysis in more detail at species level. The members at species level of these specific bacterial groups were shown in Table 26 to 30. *p* value was calculated solely on the basis of each specific group carriers.

The members of genus *Prevotella* detected by pyrosequencing in both vegetarians and non-vegetarians consisted of 4 species: *P. bivia, P. copri, P. stercorea, and P. stercorea* (Table 26). Only % relative abundance of *P. copri* was significantly different between both sample groups (p = 3.39E-07). This bacterium was very high abundance in vegetarians. Hence, it could be used an indicator for microbiota of Thai vegetarians.

Table 26 The members of *Prevotella* sp. detected by pyrosequencing

Species	N group		V group		*p* value
	% relative abundance	Carrier (subjects)	% relative abundance	Carrier (subjects)	
Prevotella bivia	0.003	2	0.001	1	-
Prevotella copri	3.804	28	16.901	34	3.39E-07
Prevotella ruminicola	0.011	1	0	0	-
Prevotella stercorea	1.566	18	1.561	24	0.571

As it was already reported, various species in genus *Bacteroides* belong to this group (Table 27). In our study *B. distasonis, B. merdae*, and *B. ovatus* were not found in Thai subjects whereas *B. caccae, B. eggerthii, B. fragilis, B. stercoris*, *B. thetaiotaomicron, B. uniformis,* and *B. vulgatus* were found in the most subjects. Except *B. fragilis* and *B. stercoris, B. caccae, B. eggerthii, B. thetaiotaomicron, B. uniformis,* and *B. vulgatus* were significant different among both sample groups (Table 28). The abundances of *B. uniformis*

and *B. vulgatus* were higher in non-vegetarians. These two species might be used as the indicators for microbiota of Thai non-vegetarians.

Table 27 The members of *Bacteroides fragilis* group

Species			
B. caccae	*B. distasonis*	*B. eggerthii*	*B. fragilis*
B. merdae	*B. ovatus*	*B. stercoris*	*B. thetaiotaomicron*
B. uniformis	*B. vulgatus*		

Source: Shah *et al*. (1989); Horn *et al*. (1992); Lui *et al*. (2003); Matsuki *et al*. (2004)

Table 28 The members of *Bacteriodes fragilis* group detected by pyrosequencing

Species	N group		V group		*p* value
	% relative abundance	Carrier (subjects)	% relative abundance	Carrier (subjects)	
Bacteroides caccae	0.538	27	0.084	13	0.005
Bacteroides eggerthii	0.570	17	0.033	7	0.026
Bacteroides fragilis	0.301	14	0.112	15	0.206
Bacteroides stercoris	0.161	9	0.004	3	0.280
Bacteroides thetaiotaomicron	0.404	33	0.130	22	0.031
Bacteroides uniformis	2.448	33	0.396	31	9.55E-05
Bacteroides vulgatus	4.493	33	1.268	32	0.016

The members of Enterobacteriaceae detected by pyrosequencing in both vagatarians and non-vegetarians in this study were bacterial species closed to *Escherichia coli, E. hermannii, Klebsiella pneumoniae, Plesiomonas shigelloides, Pantoea agglomerans,* and *Raoultella ornithinolytica* as listed in Table 29. Only two species, *E. hermannii* and *K. pneumoniae,* were significant different among both sample groups at *p* value = 0.036 and 0.027, respectively.

Table 29 The members of Enterobacteriaceae detected by pyrosequencing

OTU No.	Species	N group		V group		*p* value
		% relative abundance	Carrier (subjects)	% relative abundance	Carrier (subjects)	
301, 509, 529, 615, 748, 810	*Escherichia coli*	1.527	34	0.652	31	0.117
1	*Escherichia hermannii*	4.703	35	0.716	31	0.036
8, 629	*Klebsiella pneumoniae*	0.793	34	2.169	33	0.027
890	*Plesiomonas shigelloides*	0.147	3	0.0006	1	-
828	*Pantoea agglomerans*	0.053	19	0.121	25	0.210
51	*Raoultella ornithinolytica*	0.278	28	0.617	31	0.087

By pyrosequencing analysis, *C. leptum, E. siraeum, F. prausnitzii, R. bromii, and R. callidus* were detected in both vegetarians and non-vegetarians by pyrosequencing (Table 30). No significant differences were found in these species between both sample groups.

Table 30 The members of *Clostridium leptum* group detected by pyrosequencing

Species	N group		V group		*p* value
	% relative abundance	Carrier (subjects)	% relative abundance	Carrier (subjects)	
Clostridium leptum	0.031	12	0.023	8	0.885
Eubacterium siraeum	0.159	8	0.139	12	0.370
Faecalibacterium prausnitzii	10.635	36	10.357	36	0.835
Ruminococcus bromii	0.347	22	0.174	20	0.199
Ruminococcus callidus	0.103	19	0.085	21	0.318

2.5 The correlation of gut microbiota, personal characters and consumption behaviors

All datasets from real-time PCR and pyrosequencing were used for correlations analysis based on 3 conditions of gut microbiota vs all subjects, gut microbiota vs non-vegetarian subjects, and gut microbiota vs vegetarian subjects. The factors correlating with microbiota were BMI, age, and time of being vegetarian and some dietary behaviors including yoghurt- and eggs consumption. By bivariate correlation of SPSS software, the results were showed that only correlations of age and microbiota as well as BMI and microbiota were found.

2.5.1 Correlation between microbiota and age

The significant correlations were slightly dominated by positive correlations between *Bifidobacterium* and age of all subjects when using real-time PCR and pyrosequencing data (Figure 16). The correlations between *Bifidobacterium* and age of non-vegetarian or vegetarian groups were not observed.

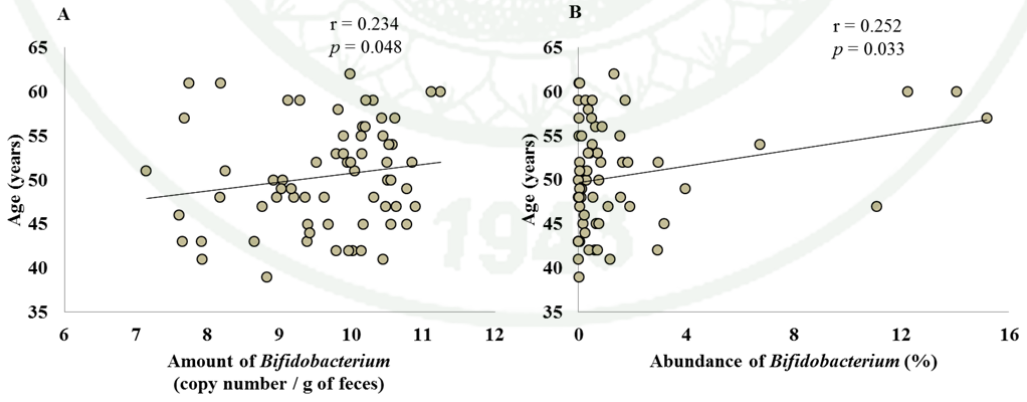

Figure 16 Correlation between age of all subjects and *Bifidobacterium* analyzed using real-time-PCR (A) and pyrosequencing at genus level (B).

Figure 17 showed the correlation between microbiota and age of non-vegetarians, the significant correlations were slightly positive with *Alitipes finegoldii*, *B. vulgatus*, and *B. uniformis* (Figure 17A, 17B, and 17C, respectively), whereas the significant correlation was slightly negative with *Roseburia faecis* (Figure 17D). There was no significant correlation between microbiota and age of vegetarians.

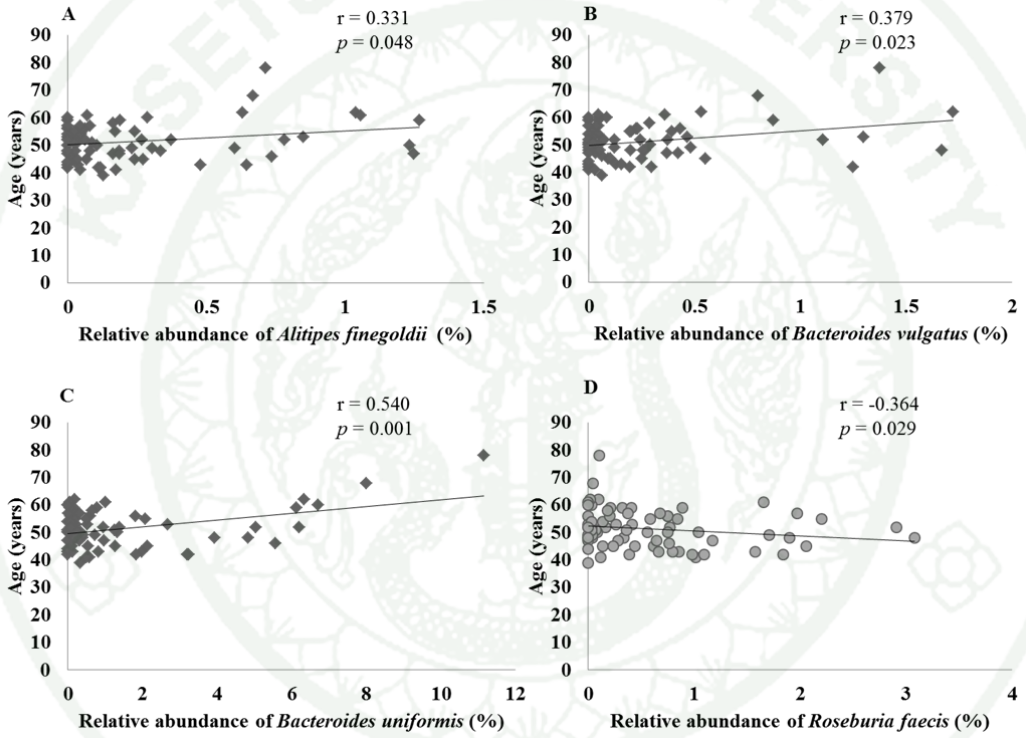

Figure 17 Correlation between *Alitipes finegoldii* (A), B*acteroides vulgatus* (B), *B. uniformis* (C), and *Roseburia faecis* (D) and age of non-vegetarians.

2.5.2 Correlation between microbiota and BMI

For pyrosequencing data at species level, the significant correlations were slightly dominated by positive correlations between *P. distasonis*, and *P. merdae* and BMI of all subjects (Figure 18A and 18B). However, *P. copri* and *Raoultella ornithinotyca* were significantly and negatively correlated with BMI of all subjects (Figure 18C and 18D).

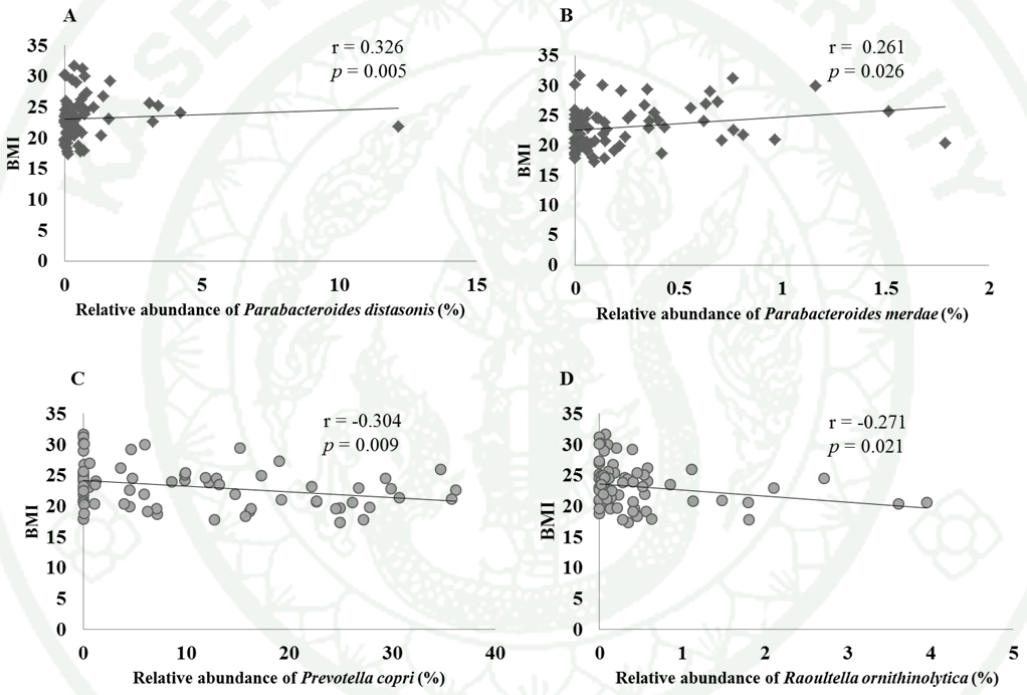

Figure 18 Correlation between *Parabacteroides distasonis* (A), *Parabacteroides merdae* (B), *Prevotella copri* (C), and *Raoultella ornithinolytica* (D) and BMI of all subjects.

Moreover, there were no significant correlations between microbiota and time of being vegetarian, yoghurt- and egg consumption. However, the correlations between microbiota and various factors were trivially.

2.6 Comparison of GI tract microbiota between human and chicken

Nakphaichit (2012) had comparatively studied cecum microbiota of 12 healthy chickens aged 28 days (D28) and chicken aged 42 days (D42). By pyrosequencing, it was shown that there was no difference in microbiota composition between these 2 age groups. Furthermore, diets of all the chickens used in this study contained only antibiotic-free plant based diets of soybean and maize which may define as vegetarian chicken. Therefore, it is interesting to compare the result of Nakphaichit (2012) with our results.

2.6.1 Family level

It seems that nearly 50% of total microbes found in chicken samples were Lachnospiraceae and approximately one third of them were Ruminococcaceae. The abundances in the family Lachnospiraceae and the family Ruminococcaceae in chicken was higher than those of vegetarians. The families Lachnospiraceae and Ruminococcaceae were one third of total bacteria found in vegetarians samples (Table 31).

Table 31 The amount of members in the family Lachnospiraceae and Ruminococcaceae in chicken samples and vegetarians

Family	Chickens (D42)	Vegetarians
	% relative abundance	% relative abundance
Lachnospiraceae	45.05	21.27
Ruminococcaceae	29.98	15.05

2.6.2 Genus level

It was noted that microbiota in chicken cecum highly differed from those of vegetarians colon as shown in Figure 19. However, its diversities were lower than those of vegetarians. The major microorganisms at genus level found in chickens were *Faecalibacterium*, *Lachnospiraceae_incertae_sedis*, *Lactobacillus*, *Ruminococcus*, and *Subdoligranulum*. The highest number found in chicken cecums belonged to *Lachnospiraceae_incertae_sedis* (approx. 30% relative abundance), while that of vegetarians was *Prevotella* (34% relative abundance). It was interesting that there was no *Prevotella* detected in chicken cecum. It was possible that there were various factors ascribed to this difference such as different physiological digestive tracts which in turn may affect the composition of microbiota.

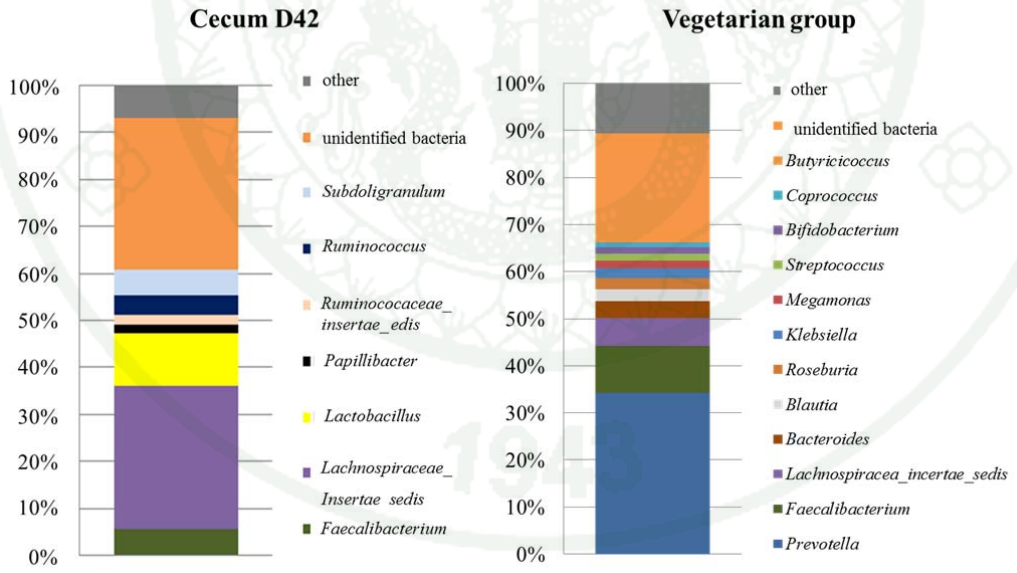

Figure 19 Gut microflora with high abundance detected in vegetarians and chickens (D42) at genus level.

The core microflora in chicken was defined as the genera which found >80% of total chickens. Consequently, only 15 out of 45 genera were regarded as core cecum microbiota in chickens (D42). These were *Anaerofilum, Anaerotruncus, Bacteroides, Dorea, Eggerthella*, *Enterococcus, Erysipelotrichaceae Incertae Sedis, Faecalibacterium*, *Lachnospiraceae Incertae Sedis*, *Lactobacillus*, *Papillibacter, Ruminocaccaceae Insertae Sedis, Ruminococcus, Streptococcus,* and *Subdoligranulum.* Only 8 genera were also the core microbiota in non-vegetarians and vegetarians (Figure 20). *Lachnospiraceae Incertae Sedis,Ruminococcus* and, *Ruminocaccaceae Insertae Sedis* in chickens were clearly higher than those in both human sample groups. The genera *Papillibacter* (found in 12 chickens), *Peptostreptococcaceae Incertae Sedis* (found in 9 chickens), *Ruminocaccaceae Insertae Sedis* (found in 12 chickens), *Subdoligranulum* (found in 12 chickens), and *Syntrophothermus* (found in 3 chickens) were found in chicken samples not in human samples.

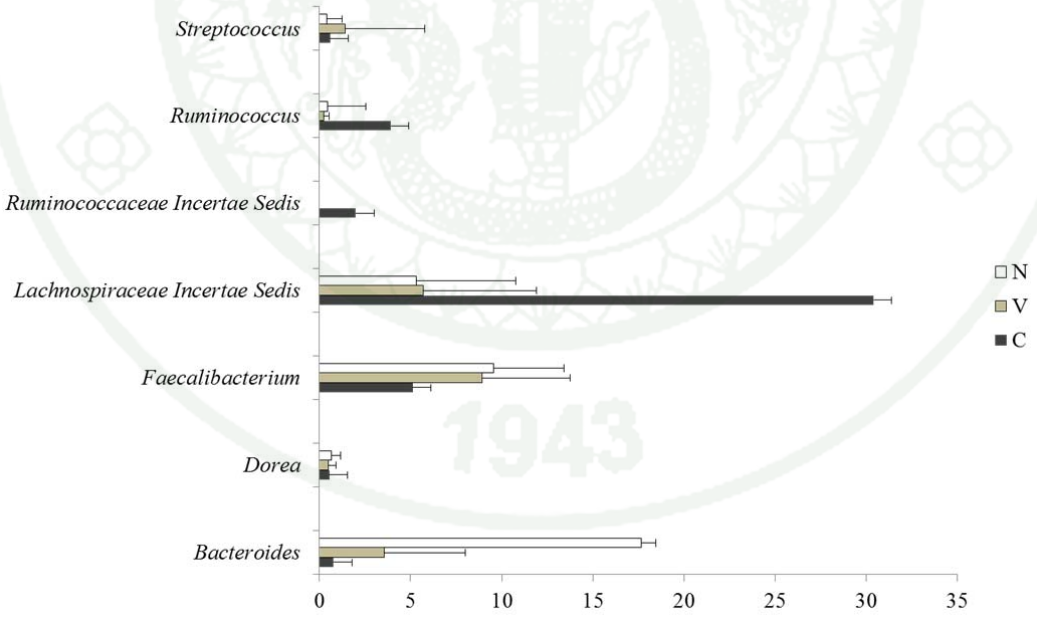

Figure 20 Gut microflora detected in both human and chicken groups at genus level found in ≥80% of total sample groups. N; non-vegetarians, V; vegetarians, and C; chicken (D42).

The datasets of all bacteria analyzed by pyrosequencing at genus level were subjected to principal component analysis (PCA). They resulted in a separation of chicken, vegetarian and non-vegetarian samples following to their specific characteristic microbiota (Figure 21). Considering to axis 1 of PCA plot, *Prevotella* and *Bacteroides* were key microorganisms for vegetarians and non-vegetarians, respectively. Besides, *Lachnospiraceae Incertae Sedis* and *Lactobacillus* were the key microbes for chickens.

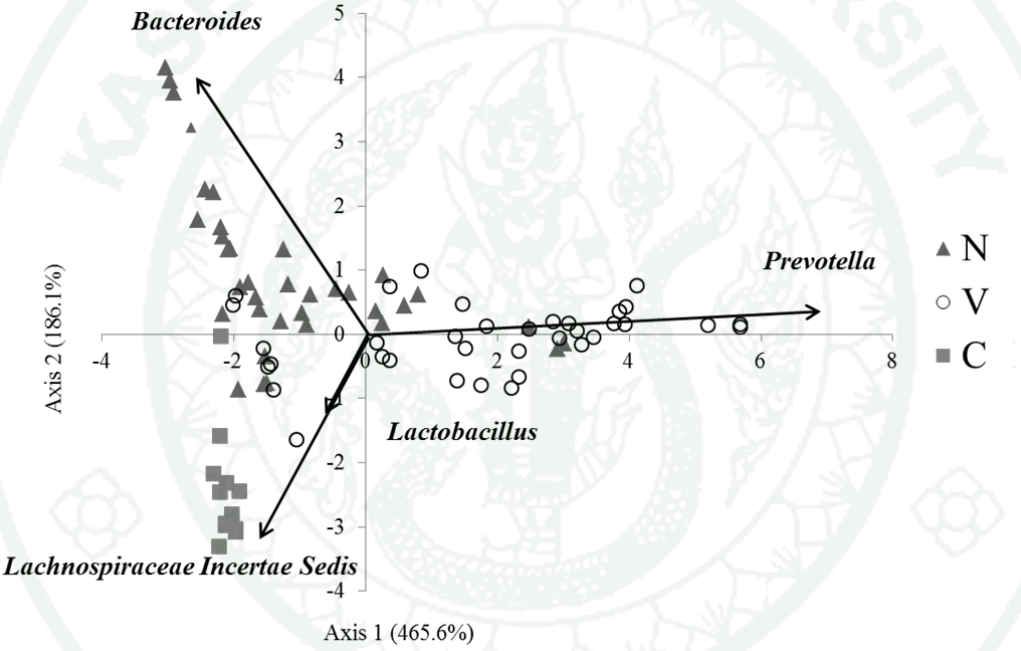

Figure 21 Species level bacterial compositions analysis using PCA plot comparison between human and chickens (D42). N; non-vegetarians, V; vegetarians, and C; chicken (D42).

2.6.3 Species level

All species identified in chicken samples (D28 and D42) and high abundances in vegetarian samples were shown in Table 32. Major species found in chickens was not observed in vegetarians. Only *F. prausnitzii* was high abundances in chickens D42 and vegetarians.

Table 32 Species identified in vegetarian and chicken samples

Species	Chickens (D42) n = 12	Vegetarians (50.9 ± 5.9 years) n = 36
Anaerotruncus colihominis	0.30	nd
Bacteroides capillosus	1.72	nd
Bacteroides vulgatus	0.00	1.27
Bacteroides xylanolyticus	0.32	nd
Blautia coccoides	4.48	nd
Blautia hydrogenotrophica	4.15	0.00
Blautia luti	1.28	0.06
Blautia producta	5.04	0.00
Blautia schinkii	1.85	nd
Blautia wexlerae	nd	1.04
Butyricicoccus pullicaecorum	1.62	nd
Butyrivibrio fibrisolvens	0.27	0.23
Clostridium aldenense	1.36	0.01
Clostridium aldrichii	0.00	nd
Clostridium amygdalinum	2.88	nd
Clostridium citroniae	0.95	0.00
Clostridium fimetarium	0.49	nd
Clostridium fusiformis	2.71	nd
Clostridium glycyrrhizinilyticum	5.46	0.00
Clostridium lactatifermentans	0.75	nd
Clostridium leptum	2.26	0.02
Clostridium nexile	2.04	0.28
Clostridium orbiscindens	2.95	0.03
Clostridium sporosphaeroides	0.66	nd
Clostridium viride	1.09	nd
Coprobacillus cateniformis	0.38	0.00
Eggerthella hongkongensis	0.50	nd
Elbe River	0.94	nd

Table 32 (Continued)

Species	Chickens (D42) n = 12	Vegetarians (50.9 ± 5.9 years) n = 36
Escherichia coli	nd	0.65
Escherichia hermannii	nd	0.72
Eubacterium cylindroides	0.54	Nd
Eubacterium eligens	nd	1.20
Eubacterium rectale	nd	3.84
Faecalibacterium prausnitzii	10.79	10.36
Germiger formicillis	nd	0.84
Hespellia porcina	nd	nd
Klebsiella pneumoniae	nd	2.17
Lactobacillus crispatus	7.61	nd
Lactobacillus johnsonii	2.57	nd
Lactobacillus salivarius	2.34	0.02
Prevotella copri	Nd	16.9
Robinsoniella peoriensis	0.74	nd
Roseburia inulinivorans	0.00	1.59
Ruminococcus albus	1.12	nd
Ruminococcus bromii	2.35	0.17
Ruminococcus callidus	1.60	0.09
Ruminococcus flavefaciens	0.29	nd
Ruminococcus gauvreauii	0.46	nd
Ruminococcus lactaris	nd	0.12
Ruminococcus obeum	1.07	0.57
Ruminococcus torques	0.45	0.53
Streptococcus thermophilus	nd	1.42
Others	11.21	33.1

nd; not detected

DISCUSSION

The microbiota of 2 groups of Thai subjects including 7 vegetarians and 6 non-vegetarians, were first analyzed by PCR-DGGE, which resulted in the classification of clusters A and B that contained the non-vegetarians and vegetarians, respectively. Subsequently, a qPCR was used for specific bacterial group quantification. From the results, cluster A contained *Bacteroides* as the major bacteria whereas cluster B contained *Prevotella* as the main bacteria. Matijašić *et al.,* 2013 also used PCR DGGE and a qPCR to study microbiota in vegetarians and omnivores in Slovenia, which revealed a higher ratio of the *C. coccoides* groupand *Bacteroides-Prevotella* group in the non-vegetarians. This study also found the *C. coccoides* group in the non-vegetarian subjects, yet it was less abundant than the *Bacteroides* group. The different types of diet and lifestyle may have been the cause of these differences in the microbiota.

Cluster A consisted of two sub-clusters; sub-cluster A1 and A2. While subjects N1, N3, N4, N5, and N6 belonged to sub-cluster A1, subject N2 belonged to sub-cluster A2. This may have been due to the singular presence of *Prevotella* in subject N2. *Prevotella* was also a typical bacteria for cluster B; the vegetarian group. The presence of *Prevotella* in subject N2 may have been due to the type of diet. From a personal interview, it was found that subject N2 consumed high amounts of fruit and vegetables (approx. 200 grams/meal). Furthermore, although no *Bacteroides* was detected in subject N2 based on the PCR-DGGE results, the real-time PCR analysis revealed a 10.2 log copy number/g of feces. The inconsistency of these two techniques may have been due to the low identity of *Bacteroides* at 96%, which was not reported in PCR-DGGE results. Therefore, the results indicated that the non-vegetarians tended to have a higher abundance of *Bacteroides* than the vegetarians. It was also noted that the PCR-DGGE patterns for subjects N3, N5, and N6 were similar. When these 3 subjects were interviewed, it was found that they always ate their meals together, which may have resulted in the similar detected patterns. Therefore, this observation implies that a similar food intake could be a major factor providing similar microbiota.

Cluster B was divided into two sub-clusters; B1 and B2. The abundant bacterium in this cluster analyzed by PCR-DGGE and qPCR was *P. copri*. *Prevotella* was previously observed in an agrarian society resident in USA (Yatsuneko *et al*., 2012). In addition, a genome analysis of *P. copri* DSM 18205 has shown that this species contains xylanase and

putative cellulase genes that may code the enzymes essential for the hydrolysis of xylan and cellulose from plant polysaccharides, respectively (National Center for Biotechnology Information. 1993, retrived on Apr 11, 2013), which in turn may have been why *P. copri* was mostly found in the subjects who regularly consumed vegetables. Sub-cluster B1 consisted of subjects V3, V4, V5, V6, and V7, all of whom were lacto vegetarians living and working at a Thai vegetarian society, whereas sub-cluster B2 included subjects V1 and V2 who lived in different places. V1 and V2 were both lacto-ovo-vegetarians who consumed different amounts of eggs at 5-6 eggs/year and 2-3 eggs/week, respectively, while the other subjects in sub-cluster B1 did not include eggs in their meals. It was also found that *R. intestinalis* and *M. funiformis* were found only in subjects V1 and V2, respectively. However, due to the low number of subjects analyzed, it was difficult to conclude that egg consumption had any effect on the bacterial types. Thus, a more extensive study is needed to clarify the effect of eggs on the microbiota.

When the numbers of samples were increased from 13 samples to 72 samples for investigation of gut microbiota using real-time PCR and pyrosequencing, the results indicated that the non-vegetarians still tended to have a higher abundance of *Bacteroides* than the vegetarians, while the vegetarians incline to have a higher abundance of *Prevotella* than the non-vegetarians. From real-time PCR results, *Bifidobacterium*, the *C. coccoides-E. rectale* group, and *Lactobacillus* were not significantly different between both sample groups. These were the same as the results in preliminary experiment and were in accordance with pyrosequencing results. In addition, Enterobacteriaceae determined in both sample groups were found in very high amount and were significantly different between both sample groups but not significantly different when analyzed by pyrosequencing. Enterobacteriaceae was previously reported to increase with age (Woodmansey *et al*., 2004). As known, the age of the subjects used in this study were about 50 years up. This might be the reason why high amounts of this family were detected. Pyrosequencing result showed that the bacterium closed to *Klebsiella*, a member of Enterobacteriaceae, was significantly different between both sample groups. This enteric bacterium was found in much higher amount in vegetarian group. This bacterium was reported to be normal flora in fruit and vegetables such as banana (Martinez *et al*., 2003), rice (Ladha *et al*., 1983; An *et al*., 2001) maize (Palus *et al*., 1996; Chelius and Triplett 2000; Dong et al., 2003), potato (Reiter *et al*., 2003) and lecttuc (Knittel *et al*., 1977). Therefore, it could be found more in the gut of vegetarians. The other significant differences were higher amounts of *C. leptum* and *F. prausnitzii-S. variabile* like bacteria groups in non-vegetarians than vegetarians. High

concentration of *C. leptum* group was due to the high amount of *F. prausnitzii-S. variabile* like bacteria group which was the member of the *C. leptum* group.

Investigation of total 72 samples of gut microbiota using real-time PCR and pyrosequencing, the results were in the same manner as the results which analyzed using PCR-DGGE and real-time PCR in preliminary experiment. The dominant gut bacterium of Thai vegetarians was *P. copri,* while that of Thai non-vegetarians was various species of *Bacteroides*. This result may be due to colonic fermentation which can inhibit some gut microbiota. The colonic fermentation results in an increase concentration of short chain fatty acids which in turn cause the decrease in pH from 6.5 to 5.5. *Bacteroides* species grew poorly at pH 5.5 (Duncan *et al*., 2009). This may be the reason why low abundance of *Bacteroides* found in vegetarians.

The most prolific genera found in the Thai subjects were *Bacteroides* and *Prevotella,* both of which have already been reported as the genera usually present in the human gut, irrespective of nation or continent (Arumugam *et al*., 2011). The present results are also consistent with the work of De Filippo *et al*., 2010; Wu *et al*., 2011. De Filippo *et al*. reported that *Prevotella* was the most prolific genus in the gut microbiota of African children from Burkina Faso, where the children were predominantly vegetarians, while *Bacteroides* was the most abundant genus detected in the gut microbiota of European non-vegetarian children living in urban Florence, Italy. Wu *et al*. also studied the link between long-term dietary patterns and gut microbiota, and found that the *Bacteroides* enterotype was highly associated with protein and animal fat, whereas the *Prevotella* enterotype was strongly associated with carbohydrate-enriched diets. Moreover, Claesson *et al* (2012) investigated the gut microbiota composition in elderly subjects and reported that healthy community-dwelling subjects consuming low fat/high fibre and moderate fat/high fibre had significantly abundances of *Prevotella* and *Ruminococcus,* whereas most of the long-term resident-care subjects consuming moderate fat/low fibre and high fat/low fibre had a high abundance of *Alistipes*, *Oscillibacter,* and *Bacteroides* (Claesson *et al*., 2012). However, the present results are in contrast to the reports of Zimmer *et al*., 2012; Liszt *et al*., 2009, which found that *Bacteroides* was the largest genus found in fecal samples of vegetarians. This inconsistency in the results could be attributed to differences in the study methods. Zimmer's study focused on the detection of *Bacteroides* and other bacteria, yet not *Prevotella,* and used a culture-based method with different selective media, including a U3G agar, Rogosa agar, DIC agar, and SPM agar. As a result, no *Prevotella* was detected in their

study. Notwithstanding, their results did show a significantly lower *Bacteroides* count in vegetarians when compared to the control group. In the case of Liszt *et al*., the primers used for the PCR-DGGE and quantitative PCR were specific to *Bacteroides* sp. and *Clostridium* sp. rather than *Prevotella,* consequently no *Prevotella* was observed. Furthermore, Kabeerdoss *et al*., 2012 quantified the fecal microbiota in vegetarian and omnivorous young women in southern India and reported that the microbial communities, especially the *Bacteroides-Prevotella* group, were similar in both the vegetarians and non-vegetarians. While these results are different from the current findings, this could be explained by the specificity of the primers used. The primers used by Kabeerdoss were specific to both *Bacteroides* and *Prevotella,* while the primers used in the real-time PCR in the present study were only specific to either *Bacteroides* or *Prevotella*.

As known, *F. prausnitzii* contains anti-inflammatory properties. Many researches were reported that *F. prausnitzii* was low amount in Crohn's disease and IBD (Sokol *et al*., 2008; Doŕe and Corthier, 2010; Clemente *et al*., 2012). From the result, this species could be detectable in high concentration in all Thai subjects. It may be possible that *F. prausnitzii* played an important role against inflammatory in the gut of all Thai subjects.

At genus level, *Akkermansia*, *Alistipes*, the bacterial species closed to *Klebsiella*, and *Parabacteroides* were significantly different but low relative abundances. Only species in the genus *Akkrmansia*, *Akkermansia muciniphila,* found in non-vegetarians had higher concentration than those in vegetarians. This species is a normalflora of human gut containing mucin-degrading property (Derrien *et al*., 2004 and 2008) and is associated with obesity and diabetes. Everarda *et al* (2013) reported that the abundance of *A. muciniphila* decreased in obesity and type 2 diabetes mice (Everarda *et al.,* 2013). In this study, *A. muciniphila* was found in 19 non-vegetarians (1.24% relative abundance on average) and 11 vegetarians (0.068% relative abundance on average). From the results, vegetarian sample group is more closely related to obesity and type 2 diabetes than non-vegetarian sample group. It might be due to low abundance of *A. muciniphila.* For species of the genus *Alistipes*, *A. finegoldii*, *A. indistinctus, A. putredinis*, and *A. shahii* were found in both sample groups. The concentration on average of these bacteria in non-vegetarians was higher than that of vegetarians. These bacteria were common human GI tract microbiota (Nagai et al., 2010; Song *et al*., 2006). Fenner *et al* (2007) reported that *A. finegoldii* was found in blood cultures from colon cancer patients (Fenner *et al.,* (2007). In contrast, *A. finegoldii* and *A. shahii* were isolated from healthy subject feces (Song *et al*., 2006). *A.*

finegoldii and *A. putredinis* can produce succinic acid and iso-$C_{15:0}$ (a long chain cellular fatty acid) (Rautio *et al*., 2003). Moreover, the long-term resident-care subjects consuming moderate fat/low fibre and high fat/low fibre had a high abundance of *Alistipes* (Claesson *et al*., 2012). The result from this study is agreed with these published articles.

In this study, Enterobacteriaceae were detected in high concentration in both sample groups when analysed using real-time PCR and pyrosequencing. The members of Enterobacteriaceae; the bacteria species closed to *E. coli, E. hermannii,* and *Klebsella pneumoniae* were found in >90% of total subjects. Some strains in Enterobacteriaceae were opportunistic pathogens such as *E. hermannii*, *E. coli, Klebsella pneumoniae* and *Pantoea agglomerans*. *E. coli* is a normal microflora in human gut and versatile enteric pathogen. It is enabled to cause diarrheal disease (Kaper *et al*., 2004). Although, rare strain of *E. coli* is an intestinal pathogen, In general, *E. coli* can produce vitamin K (Bentley and Meganathan, 1982) and B12 (Kadner, 1978). However, *E. hermannii,* and *Klebsella pneumoniae,* they are not pathogens in GI tract. *E. hermannii* is a human microflora found in stool specimen (Brenner *et al*., 1982) and an oppertunistic pathogen caused septicemia, meningitis, neonatal brain infections in weakened and/or immunocompromised hosts (Vincent, 2013). Moreover, it caused other diseases; purulent conjunctivitis (Poulou *et al*., 2008) and wound infection (Pien *et al*., 1985). When we do not wash our hands thoroughly after the excretion, then these hands touch our wound or rub our eyes, it can cause wound infection or purulent conjunctivitis.For *K. pneumoniae,* it is found as normal flora in human GI tract (Bagley *et al*., 1985). Several published papers were reported that *K. pneumoniae* can occasionally cause diarrhea especially in childhood (Ananthan *et al*., 1999; Guerrant *et al*., 1975). In addition, the concentration of Enterobacteriaceae was positively related to the amounts of *C. leptum* and *F. prausnitzii-S. variabile* like bacteria groups. It may be possible that if the members of Enterobacteriaceae caused the inflammatory in GI tract, the members of *C. leptum* group especially *F. prausnitzii* contained anti-inflammatory property. *P. agglomerans*is a plant pathogen but it can cause human infections such as infection of bloodstream, joint/bone, and urinary tract (Cruz *et al*., 2007; Jain *et al*., 2012). The prevalence of *P. agglomerans* carriers in vegetarians was higher than that of non-vegetarians due to their amounts of vegetable consumption. Although, non-vegetarians did not consume high amount of vegetables as vegetarians but non-vegetarians consumed vegetables everyday. The other enteric pathogen, *Clostridium perfringens,* is a common cause of food-borne disease, diarrhea (Meer *et al*., 1997; Veshnyakova *et al*., 2010). Twelve non-vegetarians and two vegetarinas contained *C. perfringens* in their fecal samples. It may

indicate that they took non-hygienic foods sometimes. However, there was no subject with diarrhea at least 1 month before fecal sampling in this study. Furthermore, *B. wadsworthia,* associated digestive tract disease bacterium, was detected in higher concentration and prevalent in non-vegetarians than those of vegetarians. This bacterium can induce colitis in mice and increased in ulcerative colitis patients (Devkota *et al*., 2012). From the results, it seemed that non-vegetarian volunteers have an occasion of digestive tract inflammation. The other harmful bactium, *Fusobacterium nucleatum*, is an oral microflora associated with human colorectal carcinoma (Kostic *et al*., 2013; Rubinstein *et al*., 2013). In this study, *F. nucleatum* was found in only 3 vegetarians with very low detection. From interviewing, these 3 vegetarians do not have any sign of colorectal cancer symptoms. They do not have relatives who are colorectal cancer patients.

The core gut microbiota of Thai people were *F. prausnitzii,* various species of *Bacteroides*, *P. copri* and short chain fatty acid (SCFA) producing bacteria. It was clearly suggest that *Bacteroides* and *Prevotella* enterotypes were attributions for the core gut microbiota of non-vegetarians and vegetarians, respectively. The concentration of the others specific bacterial groups including *C. coccoides-E. rectale, C. leptum* groups, *F. prausnitzii, Bifidobacterium,* and *Lactobacillus* detected in both sample groups were similar. More than 4% of relative abundance of Enterobacteriaceae was found in Thai subjects. It may cause adversely effect. This is implied that Thai people often consumed non-hygienic foods.

The correlation between *Bifidobacterium* and age of all subjects were slightly positive. It was possible that the age of all subjects were not distinct. However, the correlations between microbiota and various factors were trivially. They need more information and well experiment design.

Although, fruit and vegetables contain anti-oxidant compounds, minerals and vitamins are gainful for health. Vegetarians and vegans may particularly lack vitamin B12, D and calcium (Ca) (Craig, 2010). Nutrition balance would be a better choice to support our health.

Since diet, microbiota, and the occurrence of disease are already known to be linked, dietary modulation studies could provide value information for understanding diet-microbiota-health (Jeffery *et al*., 2013). The dietary modulation could change host's microbiota which led to a better health. Plus, the manipulation of microbiota could be useful

for medical applications. One report has suggested that the abundance of *Bacteroides* and *Prevotella* may be useful as a prognostic biomarker of disease (Wu *et al*., 2013). Thus, the *Bacteroides* and *Prevotella* counts related to different diets of the Thai subjects could provide important data as biomarker for predicting the health condition of South East Asians with similar eating styles. Some dominant species were claimed as butyrate producing bacteria such as *F. prausnitzii*. It might be used as a probiotic after other probiotic properties test. Therefore, more studies are needed on the interaction of diet modulation, the microbial community, and the occurrence of disease. However, it would be better if Thai people would be aware to their hygienic foods, nutrition balance for good healthy and benefit microbiota.

When microbiota of chicken and vegetarians were compared, chickens contained *Ruminococcus* which belonged to the family Ruminococcaceae and *Lachnospiraceae Incertae Sedis* which belonged to the family *Lachnospiraceae* in higher amount than vegetarians. It seems that nearly 50% of total microbes found in chicken samples were Lachnospiraceae and approximately one third of them were Ruminococcaceae. It means that 75% of microflora found in chicken cecum samples belonged to these 2 families, while there were one third of total bacteria found in vegetarian samples. The family *Lachnospiraceae* and the family *Ruminococcaceae* can decompost fibrolytic plant materials (Biddle *et al*., 2013). The dominant in chicken cecum samples was *Lachnospiraceae Incertae Sedis,* whereas that of vegetarian samples was *P. copri*. It was noted that microbiota in chicken cecum highly differ from those of vegetarian colon. Even though, both chickens and vegetarians did not consume animal proteins but they did not consume the same type of plant all their lifes. It was possible that their nutrient requirement, host life style or environment, endogenous substrates including digestive enzymes, mucin and other secretions from epithelial cells (Lei *et al*., 2012), pH in GI tract (pH = 5.60-5.83 in chicken cecum and pH = 5.6-6.6 in human colon) (Wilson, 2005; Gabriel *et al*., 2006), anatomical digestive tract (Duke 1986; Cummings and Englyst, 1987), cooking foods or feed processes, diversity of diet, transit-time of foods in the gut were different. These differnces may cause different microbiota in chickens and vegetarians.

CONCLUSION AND RECOMMENDATION

Conclusion

In conclusion, the present results revealed different tendencies for the microbiota in the non-vegetarians and vegetarians. The non-vegetarians tended to have a higher abundance of *Bacteroides,* while the vegetarians had a higher abundance of *Prevotella*. In addition, the non-vegetarians showed higher values of the numbers of Enterobacteriaceae, *C. leptum*, and *F. prausnitzii-S. variabile* like bacteria groups. The copy number of *Bifidobacterium* sp., *Lactobacillus* sp., and the *C. coccoides-E.rectale* group were not significantly different between the two sample groups. From the results, it clearly suggested that core gut microbiota of Thai people were *Bacteroides* and *Prevotella* enterotypes for non-vegetarians and vegetarians, respectively. The different enterotypes between both sample groups due to their eating styles. *F. prausnitzii* and *P. copri* may be used as the indicators for healthy Thai. In this study, vegetarian sample group is more closely related to obesity, type 2 diabetes, and IBD than non-vegetarian sample group due to its low abundance of *A. muciniphila,* decreasing of Firmicutes/Bacteroidetes ratio, increasing of Bacteroidetes/Firmicutes ratio. On the other hand, the non-vegetarians have a chance to be overweight subjects due to their high BMI values. The correlations between microbiota and various factors were trivially. Even if Thai gut microbiota were composed of some opportunistic pathogens but they were normal microflora which were not initiate any diseases at the present. All these results may be useful for people from South East Asia who had similar diet.

Recommendation

To succeed in determination of correlation between gut microbiota and dietary factors, it needs more information and well experimental design. The GI tract microbial function, mucosal immunity reaction should be furthur studied in more detail.

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APPENDICES

Appendix A

Questionnaire

รหัสตัวอย่าง……

โครงการวิจัย “การศึกษาข้อมูลเบื้องต้นทางจุลินทรีย์ในระบบทางเดินอาหารของคนไทย”

แบบสอบถามข้อมูลประกอบการเก็บตัวอย่าง

****

วันที่เก็บตัวอย่าง (ว/ด/ป).....เวลา

.....

ข้อมูลผู้กรอกแบบสอบถาม

<u>ตอนที่ 1 ข้อมูลทั่วไป</u>

1. อายุ …… ปี เพศ ☐ชาย ☐หญิง

2. ส่วนสูง…… เซนติเมตร (cm)

3. น้ำหนัก……กิโลกรัม (kg)

4. สัญชาติ …… เชื้อชาติ ……

<u>ตอนที่ 2 ข้อมูลด้านการบริโภคอาหาร</u>(ใส่เครื่องหมาย ✓ในช่องที่เลือก)

5. ปกติท่านรับประทานอาหารมื้อใดบ้าง (ตอบได้มากกว่า 1 ข้อ)

☐ อาหารเช้า ☐ อาหารกลางวัน ☐ อาหารเย็น ☐ อาหารค่ำ (หลัง 3 ทุ่มเป็นต้นไป)

6. ท่านรับประทาน<u>อาหารที่ทำเองที่บ้าน</u>บ่อยเพียงใด(ตอบได้ 1 ข้อ)

☐ ทุกวัน ☐สัปดาห์ละ 5-6วัน ☐ สัปดาห์ละ 3-4 วัน ☐ สัปดาห์ละ 1-2 วัน

☐ ที่บ้านไม่ทำอาหาร

7.ท่านรับประทาน<u>อาหารนอกบ้านหรือซื้อ</u>รับประทานบ่อยเพียงใด(ตอบได้ 1 ข้อ)

☐ ทุกวัน ☐สัปดาห์ละ 5-6วัน ☐สัปดาห์ละ 3-4วัน ☐ สัปดาห์ละ 1-2 วัน

☐ไม่รับประทานอาหารนอกบ้านหรือซื้อรับประทาน

8. ท่านรับประทานอาหารประเภทใดบ้าง

8.1 ☐ ทานเฉพาะอาหารมังสวิรัติ รับประทานต่อเนื่องมา.....ปี.....เดือน (ข้ามไปตอบข้อ 9)

8.2 ☐ ทานเฉพาะอาหารเจ รับประทานต่อเนื่องมา.....ปี.....เดือน (ข้ามไปตอบข้อ 9)

8.3 ทานอาหารทั่วไป (<u>แบ่งประเภทตามสไตล์อาหาร</u>) (ตอบได้ มากกว่า 1 ข้อ)

☐ อาหารไทย ☐ อาหารญี่ปุ่น ☐อาหารเวียดนาม

☐อาหารอีสาน ☐อาหารเกาหลี ☐ อาหารฝรั่ง

☐อาหารเจ ☐ อาหารมังสวิรัติ ☐อาหารฟาสต์ฟู้ด

☐ อื่นๆ (ถ้ามีโปรดระบุ) .....☐ อื่นๆ (ถ้ามีโปรระบุ).....

9.ท่านรับประทานที่มี<u>พริก</u>เป็นส่วนประกอบหรือไม่

☐มี ☐ ไม่มี

10.ท่านรับประทานอาหารที่มีส่วนประกอบของเครื่องเทศและสมุนไพรต่างๆ ใช่หรือไม่

(เช่น กระเทียม ขิง ข่า ขมิ้น พริก พริกไทย สะระแหน่ ตะไคร้ หอมแดง กระชาย เป็นต้น)

☐ใช่ ☐ไม่ใช่

11.ท่านรับประทานอาหารเสริมหรือไม่ (เช่น วิตามินต่างๆ, แบรนด์, รังนก)

☐ ไม่ได้รับประทาน

☐ รับประทาน ดังนี้ (โปรดระบุ).....

12. ประมาณ 1 เดือนที่ผ่านมา ท่านรับประทานอาหารแต่ละประเภทบ่อยเพียงใด (แบ่งตามลักษณะการประกอบอาหาร) (ใส่เครื่องหมาย ✓ในช่องที่เลือก)

ประเภทอาหาร	ความถี่ในการรับประทาน				
	ทุกวัน	5-6 ครั้ง ต่อสัปดาห์	3-4 ครั้ง ต่อสัปดาห์	1-2 ครั้ง ต่อสัปดาห์	ไม่รับประทาน
12.1 อาหารประเภทผัด (การทำให้อาหารสุกโดยใช้น้ำมันเพียงเล็กน้อย) เช่น ผัดผัก ข้าวผัด					
12.2 อาหารประเภททอด (การทำอาหารให้กรอบหรือสุกด้วยน้ำมันปริมาณมาก) เช่น ไข่เจียว, ไก่ทอด, ปลาทอด, กล้วยทอด, ปาท่องโก๋					
12.3 อาหารประเภทยำ					
12.4 อาหารประเภทต้ม /แกง					
12.5 อาหารประเภทปิ้ง / ย่าง / เผา					
12.6 อาหารประเภทนึ่ง / ตุ๋น					
12.7 อาหารประเภทน้ำพริก / น้ำจิ้ม / หลน					

13. ประมาณ 1 เดือนที่ผ่านมา ท่านรับประทานอาหารประเภทแป้งหรือคาร์โบไฮเดรตบ่อยเพียงใด (ใส่เครื่องหมาย ✓ในช่องที่เลือก)

อาหารประเภทแป้งหรือคาร์โบไฮเดรต	**ความถี่ในการรับประทาน**						
	ทุกวัน			5-6 ครั้งต่อสัปดาห์	3-4 ครั้งต่อสัปดาห์	1-2 ครั้งต่อสัปดาห์	ไม่รับประทาน
	3 ครั้งต่อวัน	2 ครั้งต่อวัน	1 ครั้งต่อวัน				
13.1 ข้าวเจ้าหรือข้าวเหนียว							
13.2 ข้าวกล้อง							
13.3 ก๋วยเตี๋ยว							
13.4 ขนมปัง							
13.5 ขนมจีน							
13.6 มันเทศ / มันฝรั่ง / เผือก							
13.7 เมล็ดธัญพืช เช่น ลูกเดือย / ถั่วต่างๆ / เมล็ดทานตะวัน / ข้าวโพด / ข้าวโอ๊ต / งาดำ/ขาว							
อื่นๆ ถ้ามี (โปรดระบุ).....							
อื่นๆ ถ้ามี (โปรดระบุ).....							

14.ประมาณ 1 เดือนที่ผ่านมา ท่านรับประทานอาหารประเภทโปรตีนบ่อยเพียงใด (ใส่เครื่องหมาย ✓ในช่องที่เลือก)

โปรตีน	ความถี่ในการรับประทาน						
	ทุกวัน			5-6 ครั้ง ต่อสัปดาห์	3-4 ครั้ง ต่อสัปดาห์	1-2 ครั้ง ต่อสัปดาห์	ไม่ รับประทาน
	3 ครั้ง ต่อวัน	2 ครั้ง ต่อวัน	1 ครั้ง ต่อวัน				
14.1 เนื้อวัว							
14.2 เนื้อไก่							
14.3 เนื้อหมู							
14.4 เนื้อปลา							
14.5 ไข่							
14.6 ถั่วต่างๆ							
อื่นๆ ถ้ามี (โปรดระบุ).....							

15.ประมาณ 1 เดือนที่ผ่านมา ท่านรับประทานอาหารประเภทเกลือแร่และวิตามินบ่อยเพียงใด (ใส่เครื่องหมาย ✓ในช่องที่เลือก)

เกลือแร่และวิตามิน	ความถี่ในการรับประทาน						
	ทุกวัน			5-6มื้อ ต่อสัปดาห์	3-4 มื้อ ต่อสัปดาห์	1-2มื้อ ต่อสัปดาห์	ไม่ รับประทาน
	3 ครั้ง ต่อวัน	2ครั้งต่อวัน	1ครั้ง ต่อวัน				
15.1 ผัก							
15.2 ผลไม้							

16. ประมาณ 1 เดือนที่ผ่านมา ท่านรับประทานอาหารดังต่อไปนี้บ่อยเพียงใด (ใส่เครื่องหมาย ✔ในช่องที่เลือก)

อาหารอื่นๆ	ความถี่ในการรับประทาน						
	ทุกวัน			5-6ครั้ง ต่อ สัปดาห์	3-4 ครั้ง ต่อสัปดาห์	1-2ครั้ง ต่อสัปดาห์	ไม่ รับประทาน
	3 ครั้งต่อ วัน	2 ครั้งต่อ วัน	1 ครั้งต่อ วัน				
16.1 นมวัว							
16.2 นมถั่วเหลือง							
16.3 นมเปรี้ยว (ระบุยี่ห้อ) ……							
16.4 โยเกิร์ต (ระบุยี่ห้อ) ……							
16.5 น้ำผลไม้							
16.6 เครื่องดื่มที่มีแอลกอฮอล์ เช่น เบียร์ / เหล้า / ไวน์							
16.7 น้ำอัดลม							
16.8 กาแฟ / ชา							
16.9 ไอศกรีม							
16.10 อาหารฟาสต์ฟู้ด เช่น แฮมเบอร์เกอร์ / แซนด์วิช / มันฝรั่งทอด หรือเฟรนช์ฟราย / ไก่ทอด							
16.11 อาหารที่มีไขมันสูง เช่น ขาหมูติดมัน, หมูสามชั้น, หนังไก่, คอหมูย่าง, แกงกะทิหรือขนมหวานใส่กะทิ							
16.12 อาหารทะเล							

<u>ตอนที่ 3 ข้อมูลด้านสุขภาพ</u>(ใส่เครื่องหมาย ✓ในช่องที่เลือก)

17. ในรอบ 1 ปี ที่ผ่านมา ท่านเคยได้รับการตรวจสุขภาพโดยแพทย์หรือเจ้าหน้าที่สาธารณสุขบ้างหรือไม่

☐ไม่เคย

☐เคย ผลการตรวจ คือ☐ปกติ ☐ไม่ปกติ

18. การตรวจสุขภาพประเภทต่างๆ ของท่านมีผลการตรวจเป็นอย่างไร

ประเภทการตรวจ	ผลการตรวจ		
	ปกติ	ไม่ปกติ	ไม่ได้ตรวจ
การตรวจระดับความดันโลหิต			
การตรวจระดับน้ำตาลในเลือด			
การตรวจระดับไขมันในเลือด			
การตรวจพยาธิ			
การตรวจมะเร็งลำไส้ใหญ่			

19.ท่านมีโรคประจำตัว หรือมีภาวะเสี่ยงต่อสุขภาพหรือไม่

●ลำไส้อักเสบ(IBD; Inflammatory Bowel Disease) ☐มี ☐ ไม่มี

☐ไม่ทราบ

(อาการ: ท้องเสียเรื้อรัง มีมูกเลือดปนในอุจจาระ ปวดท้อง น้ำหนักลด หรือผู้ป่วยบางคนอาจมีไข้ร่วมด้วย)

●ลำไส้แปรปรวน (IBS; irritable bowel syndrome) ☐มี ☐ ไม่มี ☐ไม่ทราบ

(มีอาการปวดท้อง ท้องอืด ท้องเสีย หรือท้องผูกสลับกันไป โดยไม่ทราบสาเหตุ)

●มะเร็งลำไส้ ☐มี ☐ ไม่มี ☐ไม่ทราบ

●กระเพาะอาหาร ☐มี ☐ ไม่มี ☐ไม่ทราบ

●ภูมิแพ้ ☐มี ☐ ไม่มี ☐ไม่ทราบ

● เบาหวาน ☐มี ☐ ไม่มี ☐ไม่ทราบ

●ความดันโลหิตสูง ☐มี ☐ ไม่มี ☐ไม่ทราบ

●หัวใจและหลอดเลือด ☐มี ☐ ไม่มี ☐ไม่ทราบ

อื่น ๆ ถ้ามี (โปรดระบุ) .....

20. ปกติท่านมีการออกกำลังกายบ้างหรือไม่

☐ ไม่ออกกำลังกาย

☐ ออกกำลังกาย โดยกิจกรรมที่ทำเป็นประจำได้แก่.....

ความถี่ในการออกกำลังกาย ☐ทุกวัน ☐ สัปดาห์ละ 5-6วัน☐ สัปดาห์ละ 3-4 วัน

☐ สัปดาห์ละ 1-2วัน

21. การขับถ่ายอุจจาระ (ตอบได้ 1 ข้อ)

☐ ทุกวัน วันละกี่ครั้ง (โปรดระบุ) .....

☐ 5-6วัน/สัปดาห์ ☐3-4 วัน/สัปดาห์ ☐1-2วัน/สัปดาห์

☐ น้อยกว่า 1 วัน/สัปดาห์

22. ในช่วง 1 เดือนที่ผ่านมา ท่านมีอาการท้องเสียหรือไม่

☐ มี ☐ ไม่มี

23. ท่านเคยล้างพิษลำไส้ (ดีท๊อกซ์) หรือไม่

☐ ไม่เคย

☐ เคย บ่อยเพียงใด (โปรดระบุ เช่น เดือนละ 1 หรือ 2 ครั้ง, ปีละ 2 ครั้ง) ……...

24. ยาที่ท่านรับประทานเป็นประจำ ได้แก่

☐ ไม่มี

☐ มี (โปรดระบุ) 1.ชื่อยา……รับประทาน (ปริมาณ)…...……ต่อวัน / สัปดาห์

2.ชื่อยา……รับประทาน (ปริมาณ)…...……ต่อวัน / สัปดาห์

3.ชื่อยา……รับประทาน (ปริมาณ)…...……ต่อวัน / สัปดาห์

25. ในระยะเวลา 1 เดือนที่ผ่านมา ท่านรับประทานยาปฏิชีวนะหรือไม่เช่น เพนนิซิลิน (penicillin), แอมพิซิลิน (ampicillin), สเตรปโตมัยซิน (Streptomycin), อีรีโทรมัยซิน (Erythromycin), เตตราไซคลีน (Tetracyclines), เซฟาโลสปอริน (Cephalosporin)

☐ ไม่ได้รับประทาน

☐ มี (โปรดระบุ) 1.ชื่อยา……จำนวนวันที่รับประทานต่อครั้ง (dose) ……

2.ชื่อยา……จำนวนวันที่รับประทานต่อครั้ง (dose) ……

3.ชื่อยา……จำนวนวันที่รับประทานต่อครั้ง (dose) ……

****

****

ขอขอบพระคุณอาสาสมัครที่ให้ความร่วมมือในโครงการวิจัยทั้งนี้ข้อมูลที่ได้รับจะเป็นความลับของโครงการ

Appendix B

Explanation

เอกสารชี้แจงโครงการวิจัยเรื่อง
“การศึกษาข้อมูลเบื้องต้นทางจุลินทรีย์ในระบบทางเดินอาหารของคนไทย”

โครงการวิจัยนี้ดำเนินการโดยภาควิชาเทคโนโลยีชีวภาพ คณะอุตสาหกรรมเกษตร มหาวิทยาลัยเกษตรศาสตร์ ท่านโปรดใช้เวลาอ่านข้อความในเอกสารชี้แจงโครงการและแบบแสดงความยินยอมฉบับนี้ และพิจารณาตัดสินใจว่าท่านยินดีเข้าร่วมโครงการวิจัยนี้หรือไม่ ท่านสามารถถามคำถามเกี่ยวกับโครงการวิจัยนี้กับผู้วิจัยได้ทุกครั้งเมื่อท่านต้องการ

ทำไมจึงต้องมีการศึกษาวิจัยนี้

ในระบบทางเดินอาหารหรือลำไส้ของมนุษย์ประกอบด้วยจุลินทรีย์จำนวนมาก จุลินทรีย์เหล่านี้อยู่ร่วมกันและมีความสัมพันธ์กับมนุษย์มาตั้งแต่แรกเกิด จุลินทรีย์ในลำไส้มีทั้งที่เป็นประโยชน์และเป็นโทษต่อร่างกาย สำหรับคนที่มีสุขภาพดีนั้นจุลินทรีย์เหล่านี้จะอยู่ในสภาพสมดุล จุลินทรีย์ที่มีประโยชน์จะทำหน้าที่ในการสนับสนุนระบบการย่อยอาหาร การดูดซึมสารอาหารและผลิตสารต่างๆ ที่เป็นประโยชน์ต่อมนุษย์ นอกจากนี้จุลินทรีย์ที่มีประโยชน์ยังช่วยควบคุมจุลินทรีย์ที่เป็นโทษต่อร่างกายด้วย ในขณะเดียวกันจุลินทรีย์ที่อยู่ในลำไส้บางชนิดอาจก่อให้เกิดโรคในระบบทางเดินอาหารได้อีกด้วย สาเหตุที่ทำให้จุลินทรีย์ในลำไส้เกิดการเปลี่ยนแปลงและเสียสมดุลมีหลายประการเช่น อายุและพฤติกรรมการบริโภคอาหาร เป็นต้น แม้ว่าจะมีงานวิจัยที่ศึกษาข้อมูลของจุลินทรีย์ในระบบทางเดินอาหารจากกลุ่มตัวอย่างในประเทศต่างๆ มากมาย แต่เนื่องจากจุลินทรีย์ในลำไส้ของแต่ละบุคคลแต่ละเชื้อชาติมีลักษณะเฉพาะที่แตกต่างกัน อีกทั้งการศึกษากลุ่มจุลินทรีย์ในลำไส้ของคนไทยยังไม่เคยมีการศึกษามาก่อน **ดังนั้นจึงจำเป็นต้องศึกษาข้อมูลของจุลินทรีย์ในระบบทางเดินอาหารของคนไทย** เพื่อให้ทราบข้อมูลเบื้องต้นถึงชนิดของกลุ่มจุลินทรีย์ในลำไส้ของคนไทย และเพื่อให้เกิดความเข้าใจถึงความสัมพันธ์ระหว่างกลุ่มจุลินทรีย์กับคนในวัยต่างๆ รวมทั้งความสัมพันธ์ของจุลินทรีย์กับพฤติกรรมการบริโภคอาหารของคนไทย ซึ่งข้อมูลที่ได้จากการศึกษาจะเป็นองค์ความรู้พื้นฐานต่อการพัฒนางานวิจัยทางด้านอาหารและโภชนาการเพื่อสุขภาพที่ดีของมนุษย์ต่อไปในอนาคตได้

วัตถุประสงค์

โครงการวิจัยนี้มีจุดประสงค์เพื่อศึกษาและเปรียบเทียบกลุ่มจุลินทรีย์ในลำไส้ของคนไทยในวัยต่างๆ และเปรียบเทียบกลุ่มจุลินทรีย์ในลำไส้ของคนไทยในวัยเดียวกันที่บริโภคอาหารมังสวิรัติกับกลุ่มที่บริโภคอาหารทั่วไป

เกณฑ์การคัดเลือกอาสาสมัครเข้าร่วมโครงการ

1) อาสาสมัครเข้าร่วมโครงการต้องเป็นผู้ที่มีสุขภาพดีและไม่เป็นโรคเกี่ยวกับระบบทางเดินอาหาร เช่น โรคลำไส้อักเสบหรือโรคลำไส้แปรปรวน ที่วินิจฉัยโดยแพทย์ และต้องไม่ได้รับยาปฏิชีวนะในช่วง 1 เดือนก่อนการเก็บตัวอย่าง

2) อายุของอาสาสมัครเข้าร่วมโครงการแบ่งเป็น 3 ช่วง คือ

(ก) วัยผู้ใหญ่อายุระหว่าง 20-65 ปี ที่อาศัยอยู่ในเขตภาคกลาง (กรุงเทพมหานครและปริมณฑล)

(ข) วัยสูงอายุ (อายุมากกว่า 65 ปีขึ้นไป) ที่อาศัยอยู่ในเขตภาคกลาง (กรุงเทพมหานครและปริมณฑล จ.สระบุรี และ จ.ราชบุรี)

สิ่งที่อาสาสมัครต้องทำเมื่อตัดสินใจเข้าร่วมโครงการ

ผู้วิจัยจะสอบถามข้อมูลจากอาสาสมัคร ประกอบด้วยข้อมูลทั่วไป พฤติกรรมการบริโภคอาหาร และข้อมูลด้านสุขภาพเพื่อบันทึกลงในแบบสอบถาม (เอกสารแนบหมายเลข 3) จากนั้นท่านจะได้รับอุปกรณ์การเก็บตัวอย่างเพื่อทำการเก็บอุจจาระด้วยตัวเอง 1 ครั้ง และส่งมอบให้ผู้วิจัยนำไปศึกษาเชื้อจุลินทรีย์ในลำไส้ในขั้นตอนต่อไป

ก่อนทำการเก็บอุจจาระผู้วิจัยจะอธิบายขั้นตอนการเก็บอุจจาระ (ตามแผนภาพหน้า 3) ให้ท่านฟังโดยละเอียดจนกว่าท่านจะเข้าใจและสามารถปฏิบัติด้วยตัวเองได้

ท่านจะได้รับค่าตอบแทนอย่างไร

อาสาสมัครจะได้รับค่าตอบแทนเป็นจำนวนเงินคนละ 100 บาท

มีความเสี่ยงใดบ้างที่อาจจะเกิดขึ้นต่อท่านเมื่อเข้าร่วมเป็นอาสาสมัครของโครงการวิจัยนี้

ท่านจะไม่มีความเสี่ยงใดๆ เมื่อเข้าร่วมเป็นอาสาสมัครของโครงการวิจัยนี้ เนื่องจากเป็นการเก็บตัวอย่างอุจจาระจากการขับถ่ายปกติของอาสาสมัคร โดยไม่มีการให้ยาหรือสารอาหารใดๆ แก่อาสาสมัครเข้าร่วมโครงการทั้งสิ้น

บุคคลใดบ้างที่สามารถทราบข้อมูลส่วนตัวของท่าน

ถ้าท่านตัดสินใจเป็นอาสาสมัครเข้าร่วมโครงการวิจัยนี้ ทางโครงการจะปกปิดชื่อ-นามสกุลและข้อมูลส่วนตัวของท่านเป็นความลับ จะมีเพียงคณะผู้วิจัยของโครงการเท่านั้นที่มีสิทธิ์ทราบข้อมูลของท่านเพื่อตรวจสอบข้อมูลการวิจัยได้

ท่านจะได้รับประโยชน์ใดบ้างจากการเข้าร่วมเป็นอาสาสมัครในโครงการวิจัยนี้

ท่านจะไม่ได้รับประโยชน์โดยตรงจากการเข้าร่วมโครงการวิจัยครั้งนี้ แต่ข้อมูลที่ได้รับจากการที่ท่านเข้าร่วมเป็นอาสาสมัครจะเป็นองค์ความรู้ใหม่ที่เป็นพื้นฐานต่อการพัฒนางานวิจัยซึ่งจะมีประโยชน์ต่อส่วนรวมในอนาคต

ท่านสามารถปฏิเสธไม่เข้าร่วมโครงการวิจัยนี้ได้หรือไม่

ท่านสามารถเข้าร่วมหรือปฏิเสธการเข้าร่วมโครงการวิจัยนี้ได้โดยสมัครใจ และท่านสามารถขอถอนตัวออกจากโครงการวิจัยนี้ก่อนสิ้นสุดการวิจัยได้ตลอดเวลา

เกณฑ์การแยกอาสาสมัครออกจากโครงการ

อาสาสมัครจะถูกแยกออกจากโครงการเมื่อได้รับยาปฏิชีวนะในช่วง 1 เดือนก่อนการเก็บตัวอย่าง

แผนภาพขั้นตอนการเก็บอุจจาระ

<u>การเตรียมตัวก่อนทำการเก็บอุจจาระ</u>

ท่านต้องทำความเข้าใจขั้นตอนการเก็บ (ตามแผนภาพ) เพื่อป้องกันการปนเปื้อนจากเชื้ออื่นโดยปฏิบัติตามคำแนะนำอย่างเคร่งครัดดังนี้

1) ปัสสาวะให้เรียบร้อยก่อนเก็บอุจจาระ

2) <u>ไม่เก็บ</u>อุจจาระที่ปนเปื้อนปัสสาวะหรือประจำเดือน ถ้ามีการปนเปื้อนให้เก็บใหม่ในครั้งถัดไป

3) ถ่ายอุจจาระ 1 ครั้ง แล้วแบ่งเก็บใส่หลอดตัวอย่าง จำนวน 2 หลอด ที่ให้ไป

1. วางกระดาษไว้

<u>บริเวณที่แห้ง, ไม่มีน้ำ</u>

(จุดที่ทำเครื่องหมาย ✓)

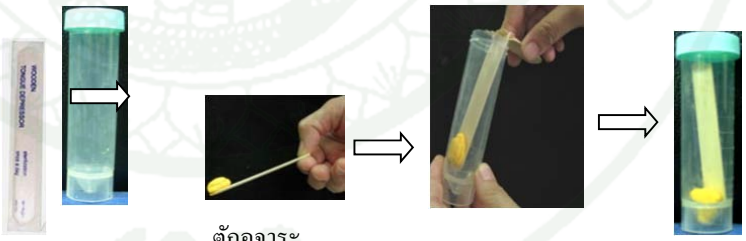
ถ่ายอุจจาระลงบนกระดาษ

2. <u>ใช้ไม้พายตักอุจจาระ</u>

<u>(ที่อยู่บนกระดาษเท่านั้น)</u>

ปริมาณเท่าหัวนิ้วโป้งใส่ลงในหลอด

หักไม้ส่วนที่ยาวเกินหลอดทิ้ง เพื่อให้ปิดฝาหลอดได้สนิท



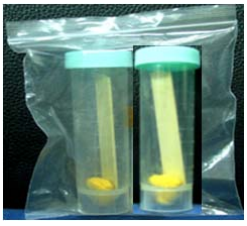
ตักอุจาระ

หักไม้ส่วนที่เกินทิ้ง

ปิดฝา

<u>หมายเหตุ</u> ใช้ไม้พาย 1 อัน ต่อการเก็บตัวอย่าง 1 หลอด

3. ใส่ถุงซิปให้เรียบร้อยแล้วนำไปแช่ในถังน้ำแข็งที่เตรียมไว้



หากท่านมีคำถามจะสามารถติดต่อได้ที่ใดบ้าง

หากท่านมีคำถามเกี่ยวกับโครงการศึกษาวิจัยนี้ ท่านสามารถติดต่อและสอบถามได้ที่คณะผู้วิจัยดังต่อไปนี้

1) นางสาว อรวรรณ ละอองคำ (นิสิตผู้ทำวิทยานิพนธ์) ภาควิชาเทคโนโลยีชีวภาพ คณะอุตสาหกรรมเกษตร มหาวิทยาลัยเกษตรศาสตร์ หมายเลขโทรศัพท์ที่สามารถติดต่อได้ในกรณีฉุกเฉิน 08-9239-4094

2) นางสาว สุภัจรี เรืองสมวงศ์ (นิสิตผู้ทำวิทยานิพนธ์) ภาควิชาเทคโนโลยีชีวภาพ คณะอุตสาหกรรมเกษตร มหาวิทยาลัยเกษตรศาสตร์ หมายเลขโทรศัพท์ที่สามารถติดต่อได้ในกรณีฉุกเฉิน 08-1426-4345

3) รศ. ดร.สุนีย์ นิธิสินประเสริฐ (อาจารย์ที่ปรึกษาวิทยานิพนธ์) ภาควิชาเทคโนโลยีชีวภาพ คณะอุตสาหกรรมเกษตรมหาวิทยาลัยเกษตรศาสตร์ หมายเลขโทรศัพท์ที่สามารถติดต่อได้ 0-2562-5089

Appendix C

Consent form

แบบแสดงความยินยอมของอาสาสมัครเข้าร่วมโครงการวิจัย (Consent form)

เรื่อง "การศึกษาข้อมูลเบื้องต้นทางจุลินทรีย์จากระบบทางเดินอาหารของคนไทย"

ก่อนที่จะลงนามในใบยินยอมให้ทำการวิจัยนี้ ข้าพเจ้าได้รับการอธิบายจากผู้วิจัยถึงวัตถุประสงค์ของการวิจัย วิธีการวิจัย รวมทั้งประโยชน์ที่จะเกิดขึ้นจากการวิจัยอย่างละเอียด และมีความเข้าใจดีแล้ว ซึ่งผู้วิจัยได้ตอบคำถามต่างๆ ที่ข้าพเจ้าสงสัยด้วยความเต็มใจ ไม่ปิดบัง ซ่อนเร้น จนข้าพเจ้าพอใจและเข้าร่วมโครงการวิจัยนี้โดยสมัครใจ และข้าพเจ้ามีสิทธิ์ที่จะบอกเลิกการเข้าร่วมในโครงการวิจัยนี้เมื่อใดก็ได้

ข้าพเจ้าเข้าใจเป็นอย่างดีว่า การวิจัยครั้งนี้เป็นการศึกษาจุลินทรีย์จากอุจจาระ และเพื่อให้เกิดประโยชน์แก่งานวิจัยนี้ ข้าพเจ้ายินยอมให้ผู้วิจัยแบ่งอุจจาระไปใช้กับงานวิจัยดังกล่าว และผู้วิจัยรับรองว่าจะเก็บข้อมูลเฉพาะเกี่ยวกับตัวข้าพเจ้าเป็นความลับ และจะเปิดเผยได้เฉพาะในรูปเล่มที่สรุปผลการวิจัยในภาพรวม

หากมีข้อสงสัยข้าพเจ้าสามารถติดต่อผู้วิจัยได้ดังนี้

1) นางสาว อรวรรณ ละอองคำ (นิสิตผู้ทำวิทยานิพนธ์) ภาควิชาเทคโนโลยีชีวภาพ คณะอุตสาหกรรมเกษตร มหาวิทยาลัยเกษตรศาสตร์ หมายเลขโทรศัพท์ที่สามารถติดต่อได้ในกรณีฉุกเฉิน 08-9239-4094

2) นางสาว สุภัจรี เรืองสมวงศ์ (นิสิตผู้ทำวิทยานิพนธ์) ภาควิชาเทคโนโลยีชีวภาพ คณะอุตสาหกรรมเกษตร มหาวิทยาลัยเกษตรศาสตร์ หมายเลขโทรศัพท์ที่สามารถติดต่อได้ในกรณีฉุกเฉิน 08-1426-4345

3) รศ. ดร.สุนีย์ นิธิสินประเสริฐ (อาจารย์ที่ปรึกษาวิทยานิพนธ์) ภาควิชาเทคโนโลยีชีวภาพ คณะอุตสาหกรรมเกษตรมหาวิทยาลัยเกษตรศาสตร์ หมายเลขโทรศัพท์ที่สามารถติดต่อได้ 0-2562-5089

ข้าพเจ้าได้อ่านข้อความข้างต้นแล้ว และมีความเข้าใจดีทุกประการ และได้ลงนามในใบยินยอมนี้ด้วยความเต็มใจ ในกรณีที่ข้าพเจ้าไม่สามารถอ่านหนังสือได้ ผู้วิจัยได้อ่านข้อความในใบยินยอมนี้ให้ข้าพเจ้าฟังจนเข้าใจดีแล้ว ข้าพเจ้าจึงลงนามในใบยินยอมนี้ด้วยความเต็มใจ

ลงนาม……ผู้ยินยอม วันที่……เดือน……พ.ศ. ……

(……)

ลงนาม……พยาน วันที่……เดือน……พ.ศ. ……

(……)

ลงนาม……ผู้วิจัย วันที่……เดือน……พ.ศ. ……

(……)

Appendix D

Certificate of approval

Ethics Committee

Institute for the Development of Human Research Protections (IHRP)

Building 8 Floor 7 Room 702 Department of Medical Science Ministry Public Health Nonthaburi Thailand 11000

Certificate of Approval

Title of Project: Preliminary Study on Gastrointestinal Tract Microbiota of Thai People. (19-2-57/Version 1)

Principle Investigator: Assoc. Prof. Dr.Sunee Nitisinprasert

Responsible Organization: Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University.

The Ethics Committee of Institute for the Development of Human Research Protections (IHRP) had reviewed the research proposal. Concerning on scientific, ICH-GCP and ethical issues, the committee has approved for the implementation of the research study mentioned above.

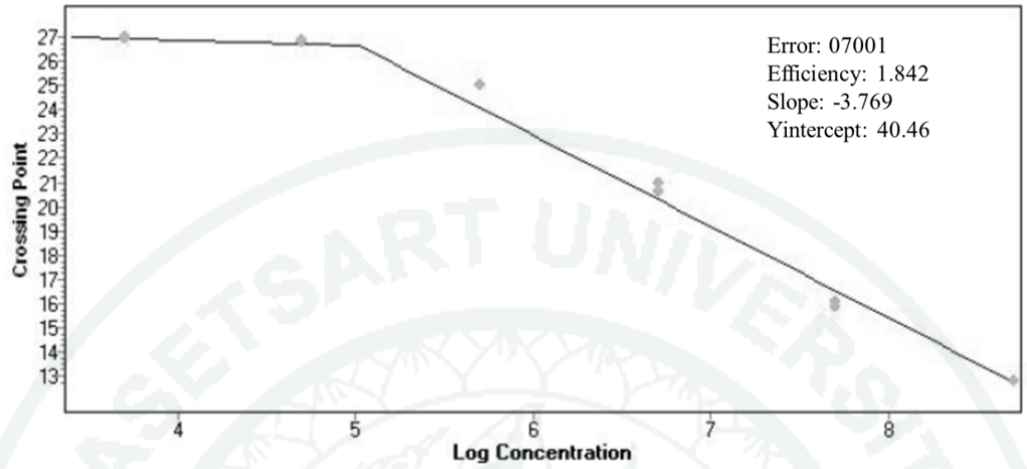
.....	.....
(Dr.Vichai Chokevivat)	(Dr.Pramote Stienrut)
Chairman	Committee and Secretary

Date of First Meeting: December 26, 2013

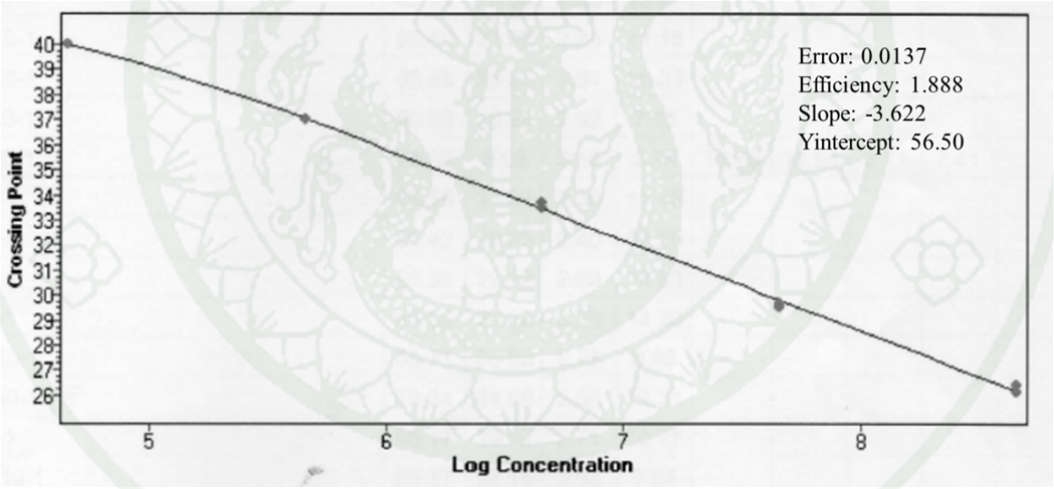
Date of Approval: February 24, 2014

Appendix E

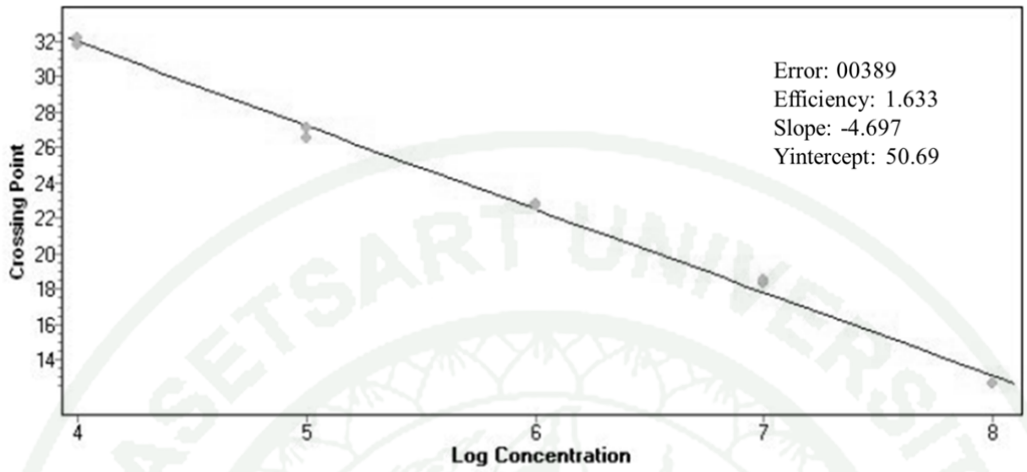
Calibration curve used for real-time PCR



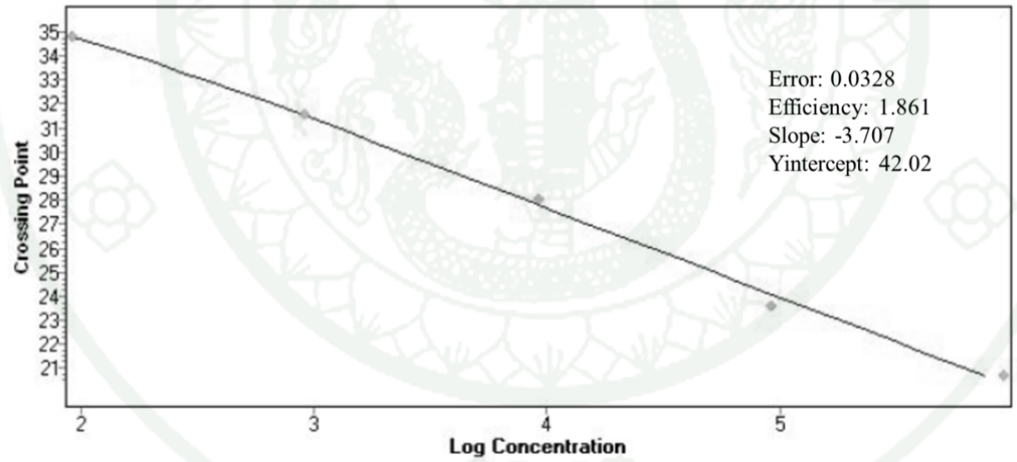
Appendix Figure E1 Calibration curve for copy number of *Prevetella* determination



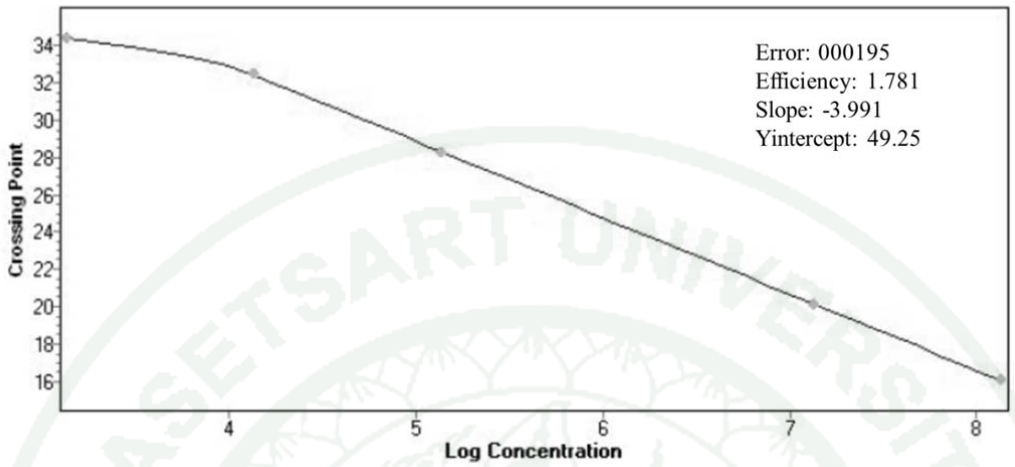
Appendix Figure E2 Calibration curve for copy number of *Bacteroides fragilis* group determination



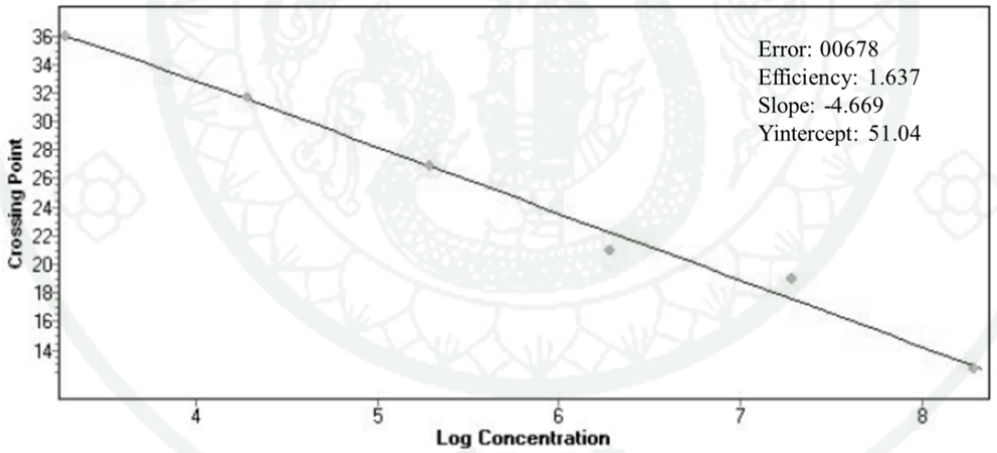
Appendix Figure E3 Calibration curve for copy number of *Bifidobacterium* determination



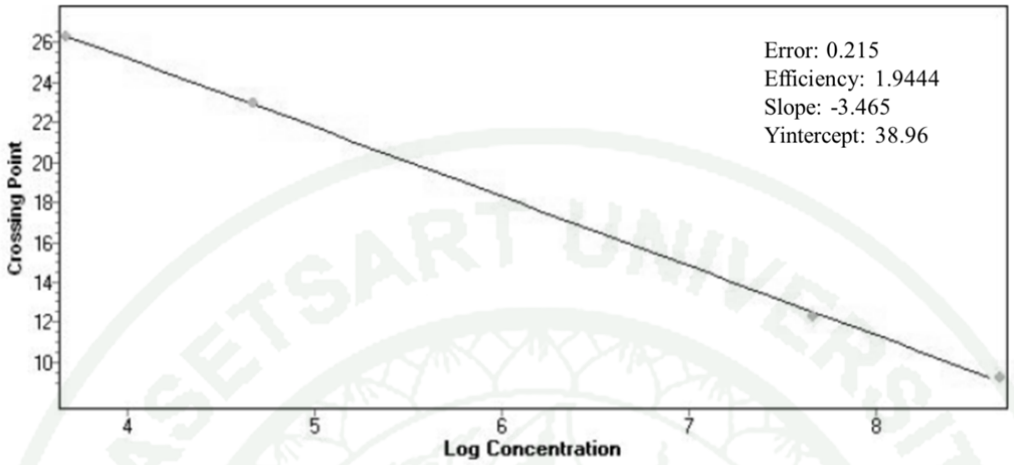
Appendix Figure E4 Calibration curve for copy number of *Lactobacillus* determination



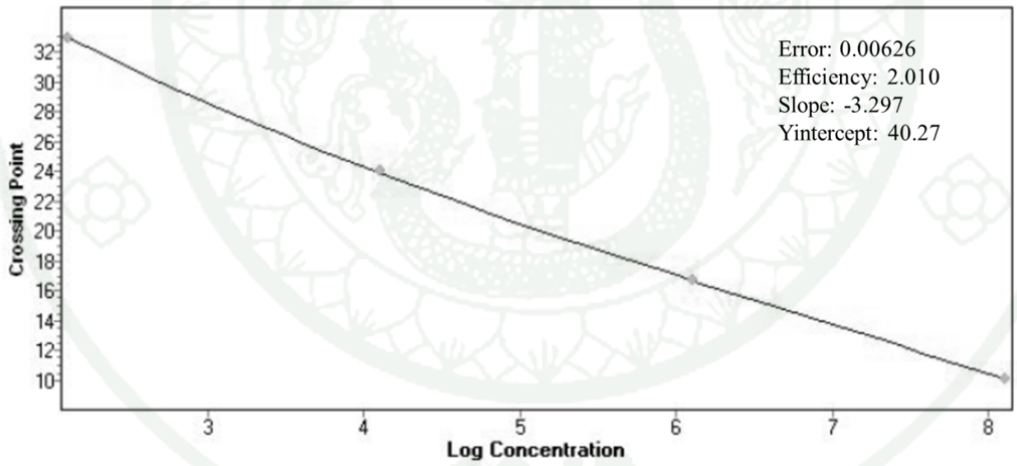
Appendix Figure E5 Calibration curve for copy number of Enterobacteriaceae determination



Appendix Figure E6 Calibration curve for copy number of *Clostidium coccoides-Eubacterium rectale* group determination



Appendix Figure E7 Calibration curve for copy number of *Clostridium leptum* group determination



Appendix Figure E8 Calibration curve for copy number of *Faecalibacterium prausnitzii-Subdoligranulum variabile* like bacteria group determination

Appendix F

Pyrosequencing results at species level

Appendix Table F1 Bacterial species found in both vegetarian and non-vegetarian groups (on average)

OTU No.	Species	% Relative abundance in non-vegetarian group	% Relative abundance in vegetarian group
450	*Acidaminococcus fermentans*	0.012	0
319	*Acidaminococcus intestinalis*	0.290	0
878	*Acinetobacter calcoaceticus*	0	0.001
455	*Acinetobacter junii*	0	0.011
250	*Actinomyces odontolyticus*	0.023	0.009
782	*Actinomyces radingae*	0.001	0.002
279	*Adlercreutzia equolifaciens*	0.034	0.005
675	*Aeromonas veronii*	0.001	0.002
25, 400, 516	*Akkermansia muciniphila*	1.243	0.068
92	*Alistipes finegoldii*	0.390	0.088
168,787	*Alistipes indistinctus*	0.109	0.019
27	*Alistipes putredinis*	0.959	0.030
468	*Alistipes shahii*	0.033	0.003
399	*Allisonella histaminiformans*	0.030	0.001
771	*Anaerococcus vaginalis*	0.003	0.001
706	*Anaerotruncus colihominis*	0.005	0
577	*Atopobium parvulum*	0.003	0.001
359	*Bacillus cereus*	0.003	0.004
38	*Bacteroides caccae*	0.538	0.084
191	*Bacteroides cellulosilyticus*	0.119	0.044
568	*Bacteroides chinchilla*	0.003	0.005
227	*Bacteroides clarus*	0.116	0.016
93	*Bacteroides coprocola*	0.292	0.019
61	*Bacteroides coprophilus*	0.663	0.057
6, 609, 785	*Bacteroides dorei*	3.026	0.563
74	*Bacteroides eggerthii*	0.570	0.033

Appendix Table F1 (Continued)

OTU No.	Species	% Relative abundance in non-vegetarian group	% Relative abundance in vegetarian group
549	*Bacteroides fluxus*	0.006	0.003
88	*Bacteroides fragilis*	0.301	0.112
113	*Bacteroides intestinalis*	0.138	0.093
41, 286	*Bacteroides massiliensis*	0.997	0.006
221	*Bacteroides pectinophilus*	0.002	0.85
66	*Bacteroides plebeius*	0.607	0.280
255	*Bacteroides salyersiae*	0.0348	0.019
728	*Bacteroides stercoris*	0.161	0.004
465, 824	*Bacteroides thetaiotaomicron*	0.404	0.130
11, 466, 716	*Bacteroides uniformis*	2.449	0.396
5, 859	*Bacteroides vulgatus*	4.493	1.268
90	*Barnesiella intestinihominis*	0.335	0.051
70, 631, 777	*Bifidobacterium adolescentis*	0.654	0.195
885	*Bifidobacterium animalis*	0	0.003
73, 413	*Bifidobacterium bifidum*	0.135	0.042
30	*Bifidobacterium dentium*	0.099	0.511
868	*Bifidobacterium kashiwanohense*	0.0139	0.124
43	*Bifidobacterium longum*	0.708	0.160
547, 811	*Bifidobacterium pseudocatenulatum*	0.129	0.316
875	*Bifidobacterium stercoris*	0.073	0.007
155	*Bilophila wadsworthia*	0.166	0.014
144	*Blautia hansenii*	0.073	0.014
415	*Blautia hydrogenotrophica*	0.018	0
476, 883	*Blautia luti*	0.090	0.060
262, 506	*Blautia stercoris*	0.022	0.039
20, 825	*Blautia wexlerae*	0.808	1.045

Appendix Table F1 (Continued)

OTU No.	Species	% Relative abundance in non-vegetarian group	% Relative abundance in vegetarian group
136	*Butyricimonas virosa*	0.174	0.018
95	*Butyrivibrio crossotus*	0.132	0.222
180	*Butyrivibrio fibrisolvens*	0.037	0.229
826	*Caldimonas hydrothermale*	0	0.004
804	*Catabacter hongkongensis*	0.017	0.001
42	*Catenibacterium mitsuokai*	0.169	0.581
872	*Christensenella minuta*	0.068	0
354	*Clostridium aldenense*	0.001	0.009
53	*Clostridium bartlettii*	0.021	0.035
165	*Clostridium bolteae*	0.412	0.018
50	*Clostridium butyricum*	0.031	0.002
333	*Clostridium citroniae*	0.028	0.004
60	*Clostridium clostridioforme*	0.412	0.193
276	*Clostridium glycyrrhizinilyticum*	0.031	0.001
423, 432	*Clostridium hathewayi*	0.028	0.005
353	*Clostridium innocuum*	0.012	0.002
442	*Clostridium lavalense*	0.014	0.002
249	*Clostridium leptum*	0.031	0.023
59, 132	*Clostridium nexile*	0.542	0.276
249	*Clostridium leptum*	0.031	0.023
59, 132	*Clostridium nexile*	0.542	0.276
111	*Clostridium orbiscindens*	0.267	0.034
270	*Clostridium paraputrificum*	0.017	0.001
216	*Clostridium perfringens*	0.037	0.007
295	*Clostridium ramosum*	0.022	0.006
458	*Clostridium scindens*	0.009	0.007
321	*Clostridium sphenoides*	0.013	0.008
512	*Clostridium spiroforme*	0.010	0

Appendix Table F1 (Continued)

OTU No.	Species	% Relative abundance in non-vegetarian group	% Relative abundance in vegetarian group
365	*Clostridium symbiosum*	0.015	0.007
414	*Clostridium tertium*	0.001	0.016
18, 158, 869, 873	*Collinsella aerofaciens*	0.951	0.637
322	*Collinsella tanakaei*	0.015	0
633	*Comamonas kerstersii*	0.002	0.003
449	*Coprobacillus cateniformis*	0.013	0
184	*Coprococcus catus*	0.075	0.094
62	*Coprococcus eutactus*	0.169	0.411
316	*Desulfovibrio fairfieldensis*	0.030	0.003
154, 310	*Desulfovibrio piger*	0.128	0.081
218	*Dialister invisus*	0.003	0.063
842	*Dialister propionicifaciens*	0	0.003
130, 274	*Dialister succinatiphilus*	0.201	0.047
123	*Dorea formicigenerans*	0.204	0.137
45, 894	*Dorea longicatena*	0.467	0.362
229	*Eggerthella lenta*	0.061	0.003
194, 350	*Enterococcus faecalis*	0.049	0.058
301, 509, 529, 615, 748, 810	*Escherichia coli*	1.527	0.652
1	*Escherichia hermannii*	4.701	0.716
33	*Eubacterium biforme*	0.526	0.533
385	*Eubacterium coprostanoligenes*	0	0.025
9, 135, 730	*Eubacterium eligens*	1.818	1.203
26, 614	*Eubacterium hadrum*	0.780	0.592
584	*Eubacterium limosum*	0.002	0.004
3, 757	*Eubacterium rectale*	2.135	3.843
97	*Eubacterium siraeum*	0.159	0.139

Appendix Table F1 (Continued)

OTU No.	Species	% Relative abundance in non-vegetarian group	% Relative abundance in vegetarian group
599	*Eubacterium sulci*	0	0.004
493	*Eubacterium tenue*	0.011	0
2, 4, 10, 12, 17, 204, 264, 390, 416, 599, 607, 608, 611, 677, 678, 844	*Faecalibacterium prausnitzii*	10.635	10.357
124	*Fusobacterium mortiferum*	0.269	0.001
773	*Fusobacterium nucleatum*	0	0.002
854	*Gemella haemolysans*	0.005	0.001
740	*Gemella sanguinis*	0.002	0
360, 406	*Gemmiger formicilis*	1.326	0.842
460	*Gordonibacter pamelaeae*	0.018	0.002
300	*Granulicatella adiacens*	0.018	0.010
156	*Haemophilus parainfluenzae*	0.029	0.155
855	*Haemophilus segnis*	0.001	0.002
511	*Holdemania filiformis*	0.0121	0.001
307	*Howardella ureilytica*	0.020	0.014
8, 629	*Klebsiella pneumonia*	0.793	2.170
31	*Lachnospira pectinoschiza*	0.864	0.382
231	*Lactobacillus acetotolerans*	0.009	0.005
253	*Lactobacillus alimentarius*	0.008	0.003
107	*Lactobacillus fermentum*	0.139	0.076
252	*Lactobacillus gasseri*	0.018	0.002
737	*Lactobacillus murinus*	0.004	0
112	*Lactobacillus namurensis*	0.036	0.008
367	*Lactobacillus nodensis*	0.004	0

Appendix Table F1 (Continued)

OTU No.	Species	% Relative abundance in non-vegetarian group	% Relative abundance in vegetarian group
589	*Lactobacillus oris*	0.001	0.005
792	*Lactobacillus plantarum*	0	0.002
272	*Lactobacillus rhamnosus*	0.020	0.018
182	*Lactobacillus ruminis*	0.012	0.127
267	*Lactobacillus salivarius*	0.025	0.023
309	*Lactococcus garvieae*	0.025	0.008
299	*Lactococcus lactis*	0.024	0.006
426	*Lactococcus plantarum*	0.007	0.002
241	*Leuconostoc citreum*	0.018	0.016
7, 623	*Megamonas funiformis*	2.114	1.636
345	*Megasphaera elsdenii*	0.044	0
203	*Mitsuokella jalaludinii*	0.075	0.005
630	*Mogibacterium diversum*	0.003	0
404	*Morus indica*	0.009	0.011
99	*Odoribacter splanchnicus*	0.253	0.061
802	*Oribacterium sinus*	0.002	0.002
340	*Oxalobacter formigenes*	0.007	0.021
828	*Pantoea agglomerans*	0.053	0.121
32, 207	*Parabacteroides distasonis*	1.218	0.174
296	*Parabacteroides goldsteinii*	0.038	0.005
645	*Parabacteroides gordonii*	0.005	0.001
87, 377	*Parabacteroides merdae*	0.431	0.072
161	*Paraprevotella clara*	0.181	0.225
172, 479	*Parasutterella excrementihominis*	0.107	0.028
856	*Parvimonas micra*	0.002	0.001

Appendix Table F1 (Continued)

OTU No.	Species	% Relative abundance in non-vegetarian group	% Relative abundance in vegetarian group
750	*Pediococcus ethanolidurans*	0.001	0
765	*Peptoniphilus lacrimalis*	0.005	0
780	*Peptostreptococcus anaerobius*	0.002	0.002
215	*Phascolarctobacterium faecium*	0.077	0.001
96	*Phascolarctobacterium succinatutens*	0.159	0.490
153	*Plesiomonas shigelloides*	0.147	0.001
890	*Prevotella bivia*	0.003	0.001
68, 175, 226, 419, 467, 613, 693, 799	*Prevotella copri*	3.804	16.903
593	*Prevotella ruminicola*	0.011	0
21, 187, 248	*Prevotella stercorea*	1.561	1.571
709	*Pseudoflavonifractor capillosus*	0.012	0.008
51	*Raoultella ornithinolytica*	0.278	0.617
58, 566, 625, 724, 819	*Roseburia faecis*	0.648	0.539
57, 388	*Roseburia hominis*	0.349	0.319
104	*Roseburia intestinalis*	0.171	0.179
16, 40, 537, 838	*Roseburia inulinivorans*	1.362	1.589
443	*Rothia mucilaginosa*	0.004	0.007
63	*Ruminococcus bromii*	0.347	0.174
145	*Ruminococcus callidus*	0.103	0.085
769	*Ruminococcus champanellensis*	0.005	0
15, 485	*Ruminococcus gnavus*	0.163	0.136
109, 598	*Ruminococcus lactaris*	0.169	0.120
75, 89, 464	*Ruminococcus obeum*	0.361	0.572

Appendix Table F1 (Continued)

OTU No.	Species	% Relative abundance in non-vegetarian group	% Relative abundance in vegetarian group
578	*Ruminococcus productus*	0.005	0.001
44, 162, 556, 581, 775, 823	*Ruminococcus torques*	0.527	0.527
338	*Slackia piriformis*	0.019	0
472	*Solobacterium moorei*	0.008	0.001
702	*Staphylococcus gallinarum*	0.004	0.001
585	*Streptococcus anginosus*	0.004	0.003
169	*Streptococcus parasanguinis*	0.078	0.032
23	*Streptococcus thermophilus*	0.315	1.424
293	*Sutterella parvirubra*	0.047	0
100	*Sutterella stercoricanis*	0.197	0.075
176	*Sutterella wadsworthensis*	0.087	0.054
436	*Turicibacter sanguinis*	0.015	0
114	*Veillonella parvula*	0.020	0.492
186	*Weissella cibaria*	0.022	0.083

CURRICULUM VITAE

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: 2009-2011 Corporation between Thailand Institute of Scientific and Technological Research and Academic Institute on Graduate Program Development

: 2010 Asian Core Program, a Japan Society for the Promotion of Science (JSPS) grant (Japan)

PUBLICATION : Chaiyawan, N., P. Taveeteptaikul, B. Wannissorn, S. Ruengsomwong, P. Klungsupya, W. Buaban, and P. Itsaranuwat. 2010. Characterization and probiotic properties of Bacillus Strains isolated from broiler. Thai J. Vet. Med. 40: 207-214.

: Ruengsomwong S, Korenori Y, Sakamoto N, Wannissorn B, Nakayama J, Nitisinprasert S. 2014. Senior Thai fecal microbiota comparison between vegetarians and non-vegetarians using PCR-DGGE and real-time PCR. J. Microbiol. Biotechnol. 24: 1026-1033.