Study on the in vivo and in vitro links between diet and gut microbial community in Thai

キスセ, ジュマ, ムッサ

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Kisuse Juma Mussa

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Abstract

The human gastrointestinal tract is the home of a microbiota that plays a vital role in enhancing host living conditions. Diet is the key influential factor among the extrinsic factors contributing to the alteration of microbial composition, structure, and diversity. Notably, recent change in dietary life in the developing countries had profound effect on the microbiota of citizens. Since the loss of traditional diets is also serious in Thailand, especially the urban area, the author aims to capture the current status of Thai gut microbiota which may link to alteration of health condition in Thai.

The cross-sectional observational study was conducted on school-aged children of urban (n = 17) and rural (n = 28) in Thailand. Their dietary records indicated that children living in urban Bangkok tended to consume modern high-fat diets, whereas children in rural Buriram tended to consume traditional vegetable-rich diets. Sequencing of 16S rRNA genes amplified from their stool samples showed that children in Bangkok have less *Clostridiales* and more *Bacteroidales* and *Selenomonadales* compared to children in Buriram and that bacterial diversity is significantly less in Bangkok children than in Buriram children. In addition, fecal butyrate and propionate levels decreased in Bangkok children in association with changes in their gut microbial communities.

Then, the author investigated the effect of staple food in Thailand, namely rice, on the gut microbiota by using in vitro culture model. The author focused on glutinous rice that has composed one of Thai traditional dietary culture, notably in north and northeast parts of Thailand. An in vitro mix culture of fecal bacteria community was performed in a single batch fermenter with pH control. Two different types of fecal microbiota were inoculated to media added by rice powders respectively prepared from sticky rice and

jasmine rice and were incubated anaerobically for 24 h. The microbiome and short-chain fatty acid (SCFAs) production were monitored. In both media added by jasmine rice powder and sticky rice powder, lactate sharply increased after 12h with an increase of Bifidobacterium, whereas it was not observed in the control batch without the addition of rice powder. This result suggests that *Bifidobacterium* grew with lactate fermentation using rice carbohydrate. On the other hand, *Bacteroides* increased in the control batch and sticky rice batch, but not in jasmine rice batch, suggesting that *Bacteroides* prefers sticky rice to jasmine rice. The promotion of *Bifidobacterium* growth is expected to benefit the host health, while the health benefit *Bacteroides* increase requires further study.

This thesis shows an on-going effect of dietary modernization on the human colonic environment. It is indispensable to evaluate the functionality of each food in respect to the benefit to gut microbiota in addition to direct host benefit. To gain this insight, continue of in vivo and in vitro studies is important.

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Juma Mussa Kisuse

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Chapter 1

General introduction

1.1. The gut microbiome

The microbiome is a collective term for the community of the microorganism resides in or on a particular environment of the living organism, such as the human body, which includes a population of bacteria, viruses, fungi, protozoa, etc. [1]. The human gastrointestinal tract (GIT) is the home of a microbiome that plays a vital role in enhancing host living conditions. The GIT microbiome is acquired at birth and increases its diversity as human growth while shifting depending on the external and internal factors [1]–[5]. Diet is the key influential factor among the extrinsic factors contributing to the composition and diversity of gut microbiota [1]–[3].

Firmicutes and *Bacteroidetes* are the major dominant phyla in human GIT microbiome, each including several families. However, *Bacteroides*, which belongs to *Bacteroidetes*, tends to dominate GIT microbiome of people in the developed countries at the genus level as compared to most abundant *Firmicutes* genera [6]–[8]. Human microbiome are clustered as enterotypes based on the composition of the microbiome structure. Enterotypes stand as community types of gut microbiome cored by dominant taxa, *Prevotella*, *Bacteroides*, and *Ruminococcus*, respectively [9]. However, enterotypes by *Bacteroides* and *Ruminococcus* are not always observed as discrete clusters while *Prevotella* enterotype is observed widely over the world as the distinctively high abundance of this and related genera [10], [11]. Further, enterotypes are more or less consistent in individuals but changeable over time if a cause happens [11].

Gut microbiota benefits the host by its significant role of short-chain fatty acids (SCFA) fermentation on non-digestible substrates like dietary fibres from carbohydrates [12], [13]. Some of these facilitate energy balance in the host through enhancing calories harvesting, consuming, and storing in the body for future use [14]. Not

limited to SCFA production, but also indolepropionic acid, correlate with reducing risk of type 2 diabetes [15], [16].

Human beings and other animals' benefit from hosting gut microbiome. The linking between commensal microbial community and secretory Immunoglobulin A (IgA) helps to shape the microbial community, prevent mucosal infection together with follicular helper cells (Tfh), e.g., T-helper 17 (Th17) which confer mucosal immunity against invading enteropathogens [17], [18].

The gut microbiome provides several benefits as explained before, hence researching them is essential to ensure the better way that hosts can manipulate microbiome and their bio-products and gain advantage from their fermentative function.

1.2. Link between diet and gut microbiome

Diet, with its entirety composition of energy and nutrients in foods and beverages consumed regularly by people [19], have profound impacts on their gut microbiome composition and diversity [1]–[3]. The gut microbiome's digestive ability on different food components shape up its structure and increases its metabolic activity as well as rendering the host energy increase available and, enhancing the high capacity of its storage [20]. Different types of diets are being consumed worldwide not only for energy and nutrient intakes but also as a part of the cultural activity or for the purposes of shaping up body. The varieties dietary habits include the Paleo diet, Vegetarian diet, Vegan diet, Mediterranean diet, Chinese herbal diet, Washoku diet, Microbiotic diet, Ketogenic diet, and Western diet. Some of these dietary habits offer varieties of minor and major nutritional components that significantly influence host well-being, even

partially for specific population or specific occasion [78]. The digestion of simple and complex sugars, as well as resistance starches, is active in the descending part of GIT with ultra-dense microbial community consisting of the highest variety of species among human microbiome [21]–[23],[74]. However, a diet rich in fat causes a decrease in *Bacteroidetes* families *Bacteriodaceae, Prevotellaceae*, and *Rickenellaceae* with the increase of *Clostridiaceae* [24]. Likewise, it is well documented that the ratio of *Bacteroidetes* to *Firmicutes* are reduced, while the proportion of *Enterobacteriales* and *Escherichia coli* significantly increases in animals fed a high-fat diet. [25] Elsewhere, the protein-rich diet was found to show a mixed effects on the gut microbiome through its metabolites [26].

In general, end products of dietary digestion, via the action of the microbiome, provide necessary nutritional benefits to the host, as well as determine the intestine's health. These effects can be the consequence of short-term or long-term interventions leading to transient or permanent host transformation.

1.3. Gut microbial metabolite and significant role of short-chain fatty acids (SCFAs)

Gut microbial metabolites have a great impact on the organism's health. They can be evaluated by fecal metabolites which represents the end products of food molecules in GIT [27]. Wide range of diets including carbohydrates, proteins, lipids as well as some plants phenolic compounds contribute to the production of the known varieties of metabolites. These metabolites include major short-chain fatty acids (SCFA), vitamin production, amino acid (AAs) synthesis, branched-chain fatty acids (BCFA) e.g.,

Iso-valerate and Iso-butyrate. Moreover, some minor metabolites like phenolics, trimethylamine N-oxide (TMA), choline, betaine, carnitine, biogenic amines are included in the metabolites composition produced [28], [29].

The SCFAs are highly produced in the GIT microbiome and rapidly absorbed to the host and thereafter voided in the feces. The major fecal SCFAs are acetate, propionate, and butyrate and they are detected with the ratio of 60:20:20 although the ratio varies substantially depending on individual microbiome and consumed diets [81], [82]. Acetate and propionate are produced in the small and large intestines, while butyrate is found mainly in the cecum and colon [30]. Acetate is produced in a variety of biosynthetic pathways including synthesis from hydrogen and carbon dioxide or formic acid by acetogenic bacteria as well as carbohydrates fermentation performed by a large variety of intestinal bacteria [31]. Several members of the clostridial clusters IV and XIVa in GIT, including, *Roseburia* species, *Lachnospiraceae* species and *Faecalibacterium prausnitzii* are butyrate producers, while propionate producers are scarce with mainly involving of clostridial cluster IX [31], and few *Bacteroides* and *Prevotella* species [32].

SCFAs maintains the homeostatic environment of the gut by providing energy to the colonic epithelial cells [12], [13]. Also, they help to preserve intestinal barrier through regulating the expression of tight junction proteins [30], and, help to control lipid and glucose metabolism [33], [34]. Moreover, SCFAs are involved in activation of GPR41 and GPR43, leading to the secretion of chemokines and cytokines, which mediate protection of tissue inflammation on the intestinal epithelial cells [35]. On the other hand, butyrate inhibits histone deacetylase (HDAC) related to suppressing malignant transformation and removing precancerous cells in the colon through apoptosis [36]. Altogether, SCFAs play an important role in maintaining the intestine's health condition

while making balance of gut microbiome. Major SCFAs produced are mostly achieved by metabolic cross-feeding, such as converting acetate to butyrate. The metabolic cross-feeding can also be achieved by propionate producing bacteria of clostridial cluster IX [31]. However, significant imbalance of gut microbiota, the status so-called “dysbiosis”, is accompanied by low SCFA production, therein gets rid of these benefits of SCFAs and leads to non-communicable diseases [31], [37]–[39].

Diet rich in vegetables, fibers, and fruits benefits the consumer by establishing better health linking with increased beneficial gut microbiota and production of diversified microbial metabolites to play a great role in decreasing gastrointestinal disease e.g. colon cancer, celiac sprue and metabolic diseases such as diabetes mellitus.

1.4. Impact of Thai diet and Western diet on health of the host

1.4.1. Thai dietary habits

Diet style differs among the four regions of Thailand, namely northern, north-eastern, central, and southern [76]. Like other Asian countries, Thai eat rice as a staple food supplying a major source of carbohydrates. This complies with the recent findings, which indicated a high frequency of rice consumption to the Thai children as compared to children from China and Japan [40]. In northern and north-eastern Thailand, people consumed steamed glutinous, and fermented rice as well as non-glutinous rice, while people in central and southern area mainly eat steamed rice [77]. The dietary dishes in different regions of Thai carry different compositions as well as taste [78]. It is reported that high vegetables and low in fat were the main characteristics of Thailand's traditional cuisines [40]. In northern and southern dishes tend to be spicy, while being milder with

added sugar, coconut milk in northern area. In central area, many dishes are flavoured with herbs, sugar, and coconut milk [79], [80]. Thai curry is world famous menu but differs in compositions and taste among areas [78]. It is also reported that there is a possible anti-tumour promoting properties of traditional Thai food items and some of their active constituents [41].

Food modernization has taken a toll on the Thai diet in which there are marked increased consumption of fat, sugar, and animal proteins [42]. The effect of an increase in fat consumption associates with some metabolic diseases like diabetes [43]. The growth of the retailing market became mostly dominated by the modern "supermarket and convenience stores and causes decrease of tradition food consumption in Thai people" [44]. Likewise, there was an increase in expenditure on Western food in Thailand by 40% from 1999 to the year 2005 [83]. The data indicate that the affected regions concentrated urban areas, whereas people in rural still keep the traditional dietary composition, although reported to start leaning to the West [45].

To improve the nutritional status citizen, Thai started documentation of all nutritional data in Thai Food Composition Database [84], while, providing education to its citizen on the beneficial dietary practices [46]–[48].

1.4.2. Impact of Western diet on health of the host

Western Diet (WD) can be characterized high in fat, sugar, animal protein and low in dietary fiber in its composition. There is a report showing such WD damages gut microbial community, within a short time intervention in humanized mice [49]. Moreover, increased consumption of WD, which contains excessive refined sugars and,

red meats and less fruits, and vegetables, is associated with systemic inflammation leading to the high risk of several metabolic diseases, e.g. obesity and diabetes mellitus [50], [51]. Since WD contains high lipids, it enhances the deposition to visceral adipose. Surging the amount of adipocytes in blood, sends, a false alarm to the brain to releases inflammatory substances including interleukin (IL) 1, IL-6 and tumor necrosis factor (TNF) over time [52], [53], and the body has a low response to the same kind of alarm/infection due to inflammatory substances being deactivated/silenced. When the infection real happens, the body delays in releasing response, and hence infection is acquired. Therefore, the overconsumption of WD upturns inflammation resulting in the increase of risk of both communicable and no-communicable disease [52], [54].

The summary of the microbiome changes following the intervention of WD ranges depending on the condition of a subject is exposed on. The transition of Thai dietary style to the West, influences nutrient balance of consumers but also their gut microbiome [42], [45], [55]. A recent study on the Thai people who migrated to US showed a marked increase in *Bacteroides* with a decrease in *Prevotella* [56]. Apart from dietary interventions, increase or decrease in some bacteria species supposedly could be used as an important biomarker for disease diagnosis as well as in prognosis in a near future.

Gut microbial community biosynthesizes a series of vitamins, which act as co-enzymes assisting in glucose, fat, and protein metabolism as well as RNA and DNA synthesis [57]. WD may hamper the vitamin biosynthesis by gut microbiome. It is also known that dietary emulsifier frequently used in processed food have adverse effect through the increase of bacterial translocation across epithelia, which may link to increased societal incidence of obesity/metabolic syndrome and other chronic

inflammatory diseases [58]. Altogether, overconsumption of WD high risk to promote any disease with both short-term and long-term interference on the gut microbiome [58].

1.5. Gut microbiome of Thai community

The microbiome of the Thai population has been documented in different age groups by several researches [40], [45], [55], [59]. Briefly, the Thai population has two enterotype-like microbiome community, each defined by high abundance of Prevotella (P-type) or high abundance of *Bacteroides* and *Bifidobacterium* (BB-type). The report showed that children in urban Bangkok harbored BB-type while rural children in rural Khon Kaen had P-type, suggesting that Thai people is in the transition state at the enterotype phase from P-type to BB-type [40]. This observation complies with their dietary status being modernized from urban to rural. The shift in enterotype phase was also observed in a study investigating gut microbiome of vegetarians and non-vegetarians in Bangkok in which vegetarians mostly harboured P-type [55]. These findings suggest that P-type favours plant-based diets. Moreover, recent study, which will be presented in this thesis, emphasized the impact of modern diet with findings that gut microbiome of urban children has lower bacterial diversity with less amount of SCFA productivity compared to rural children [45].

1.6. In-vitro fermentation model for gut microbial community

An in-vitro culture of gut microbial community can be employed to monitor the real-time bacteria growth as well as metabolism in the community. It may range

from batch to dynamic fermentation models [60]. It may start from unknown community of bacteria such as fecal sample or known bacteria mixture, both being monitored by any systems including real-time PCR or next generation sequencer (NGS) [61]. Alternatively, human trials always provide straightforward results but is accompanied by the invasive examination and frequent and excess sampling [62]. Also, human trial always suffers from big variance due to different background of participants, not like model animal experiments, which obliges the researchers to study using a large number of subjects. In-vitro fermentation model has a potential to substitute such human study with fast and high-efficiency outcome under many parameter-controlled experiments [63]. Furthermore, enrichment and isolation of bacteria from microbiome are available, notably using different set of substrates in the fermentation [64]–[68].

However, in-vitro studies are not free from errors and challenges that include slow substrate digestion or complete stop of metabolism of substrates on the presence of inoculum [62] the coupling of metabolites, [69] which might affect result stability and reproducibility of the study.

Also, in vivo culture model always validates the findings in-question on how the medium in fermenter reflects real physiological condition of host. One of the goals of in vitro model is to realize the microbiome community in fermenter with same composition and same metabolic activity as fecal inoculants. Next generation sequencer (NGS) and LC-MS or NMR are powerful tool to compare microbiome structure and metabolic activity between in-vitro and in-vivo samples [70].

1.7. Aims and outlines of this doctoral thesis

The dietary transition in Thailand has reported affecting the consumer's health [71]. The author has attracted interest in the importance of traditional local food over the influence of modern foods on young Asians' intestinal microbiome for better understanding the effect of urbanization on Thai diets. To address this notion, the comparative cross-sectional study in urban (Bangkok) and rural (Buriram) cites in Thailand was conducted. The influence of the diet was measured in terms of structure and functionality of the gut microbiome and metabolome configurations.

Rice was the major carbohydrate source consumed in the rural and urban Thailand population. They consumed mainly steamed white rice but steamed glutinous/sticky rice as well that is predominantly consumed in north and northeast parts of Thailand. Rice constitute the main food components in their diet. The effect of sticky rice in the consumers in comparison to white rice is yet to be established. Hence in this thesis, the author intends to elucidate their importance by measuring the microbiome and SCFAs composition produced in the in vitro culture model as indicators of the health.

1.8. References

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Chapter 2

Dietary habits changes in Thailand as linked to modern diets

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2.1. Abstract

Diet is the main external factor influencing the well-being of the host during the relation with gut microbiome. Thai diet, notably in the northeast region called Isan, had characteristically been rich in carbohydrates and vegetables with low in animal fats and proteins. Economic development has made a dramatic shift in lifestyle in Thailand where urbanization comes to play a significant role to establish urban lifestyle including the introduction of urban foods with different nutritional profiles. Our dietary investigation in this study showed that Bangkok children consumed protein-rich foods, much coming from processed meat, and beverages probably containing a high concentration of simple sugar, and Buriram children still mainly consumed rice and vegetables, while both cities had a high frequency of rice consumption, indicating rice to be their primary carbohydrate source. As a result of dietary nutrient estimation, Bangkok children had consumed more fats and sugar, whereas Buriram children had more dietary fibers and beta-carotene. These findings suggest that Bangkok's children's diet has become modernized and westernized while the diet of Buriram children keeps the essence of Thai traditional foods. Hence, compared to Bangkok children, Buriram children are expected to benefit much from their traditional style foods, notably in conjunction with intestinal short-chain fatty acid fermentation.

2.2. Introduction

Thailand is endowed with wealth of agricultural products both in quantity and diversity, which has been supporting health and wellbeing of Thai people from the past to present [1]. Thai diet is traditionally cereal-based and rich in vegetables, which had achieved low calorie and less fat healthy style [2]. Further, research has shown the Thai diet carries great recipes associated with lower rates of carcinogenic mutation [10]. While Thai traditional edible plants eaten as nutrition purposes might contain several of anti-tumor compounds at a high ratio [3].

Following drastic urbanization due to the economic growth in the past half-century, diets in Thailand have been largely exchanged by western diets, resulting in a notable rise in fat, sugar, and animal protein content [2]. Therein, the diets transformation is linked to increases in a dietary related disease like heart diseases [4], obesity [5], and diabetes [6] in the Thai community.

Westernization of the Thailand diet is affected by the growth of modern retailing systems such as supermarkets and convenience stores, which control half of the retailing system in Thailand [4]. Likewise, in comparison with other developing countries, in Thailand, there is an increase in expenditure of a country in the western diet food-style by 40% to the year 2005 from 1999 [11]. These reports show that the western diet influences Thai food culture into more processed and energy-dense western diets [4].

It is essential to evaluate the impact of modern diets on Thai by studying the urban (Bangkok) and rural (Buriram) in relation to gastro-intestinal microbiome study in the next chapter. The set of data are provided to identify the extent to which the society has adopted or influenced by urbanization.

2.3. Materials and methods

2.3.1. Study population characteristics

To estimate the number of subjects satisfied a statistical power in this study, we computed the PERMANOVA power based on the variance and difference of fecal microbiome data obtained in our previous study of Thai children. We used the micropower R-package [7] in which the weighted UniFrac distance matrix was simulated based on within-group distance variance (mean and standard deviation) of the data from 26 Bangkok children and therein a range of effect sizes were generated by incorporating a range of group differences. Using the simulated matrices, the PERMANOVA powers were calculated for varying effect sizes (ω2) and sample sizes. As a result, 10 subjects per group afford 80% power to detect a ω2 of 0.10, which corresponds to the difference between the Bangkok children and children in Khon Kaen, a rural city in Thailand. Ten subjects per group afford 80% power to detect an ω2 of 0.05, which corresponds to the effect observed in a controlled-feeding study [8], and 15 subjects per group afford 100% power to detect the same effect size. Accordingly, we aimed more than 15 subjects per group.

We recruited Thai children who were born and raised in Bangkok or Buriram city, and aged 9–11 years. Subjects who were administered antibiotics for 2 weeks prior to sampling or suffered from diarrhoea for 1 month prior to sampling were excluded. Eventually, we used 17 children from Bangkok and 28 children from Buriram for this study. (Table 2.1.)

Table 2.1. Characteristics of participants in Thai study

	Bangkok (n = 17[a])	**Buriram (n = 28[b])**	**p[c]**
Age (y)	10.47 ± 0.72	9.79 ± 0.63	<0.05
Gender (male/female)	13/3	12/16	<0.05
Height (cm)	145.4 ± 11.1	139.5 ± 7.4	0.11
Weight (kg)	45.6 ± 12.2	34.4 ± 8.0	<0.05
BMI (kg/m2)	21.3 ± 4.0	17.4 ± 2.9	<0.05

[a] Dietary record were missing from two samples while one sample was missing for gender information. [b] Two samples were missing for two samples and three samples were missing for metabolome analysis. [c] Statistical analysis was carried out by Welch's t-test. Genders statistical analysis was performed by Fisher's exact test [12].

Fresh stool samples were collected from these 45 children and were subjected to the 16S rRNA gene sequencing and metabolome analysis as described below. The parents/guardians reported 1-week dietary records as described below as well as answered a questionnaire that addressed the children's physiological characteristics. Welch's t-test was performed using the Excel t-test function (Microsoft Excel 2016). There were statistical differences in age, gender, weight, and body mass index (BMI) between children in Bangkok and Buriram.

2.3.2. Dietary information recording

Diets of children participating in this study were recorded by children's parents/guardians, using a dietary record form that asked about the menu, ingredients, and quantity of every meal, including breakfast, mid-morning snack, lunch, mid-afternoon snack, dinner, and after-dinner snack for 7 days before stool samples were collected. Using the INMUCAL-Nutrients V3 database NB1 program (Institute of Nutrition, Mahidol University, 2013), recorded foods were categorized and their energy (kcal) and nutrient contents (g, mg, or μg) were estimated. The energy from each food group and its fat portion were calculated per day per person and subjected to Wilcoxon rank-sum test in R ver. 3.3.2 to determine the statistical significance of differences between children in Bangkok and Buriram. Each nutrient group consumed by an individual was also calculated per day and subjected to the Wilcoxon rank-sum test to determine the statistical significance of differences between children in Bangkok and Buriram.

2.4. Results

Based on the 7-day dietary records, we summarized the daily diets of children in Bangkok and Buriram as shown in (Figures 2.1B–D). Buriram children consumed more rice and vegetables, whereas children in Bangkok consumed more bread, meat, and beverages. Notably, consumption of vegetables in Bangkok children was considerably reduced corresponding to 1.0% of total calorie intake, whereas Buriram children consumed them almost every day corresponding to 7.3% of total calorie intake. All of the participating children in both cities consumed rice, particularly steamed rice, almost every day, suggesting that rice was the main carbohydrate source for Thai children. However, its frequency and amount consumed were much greater in Buriram children (10.2 times per week, 207.1 kcal/day) than in Bangkok children (6.3 times per week, 140.8 kcal/day). Furthermore, Buriram children consumed more glutinous rice (101.4 kcal/day), served in the traditional Isan style including rice and spiced fermented fish, than Bangkok children (13.1 kcal/day). On the other hand, Bangkok children consumed more single-dish rice (94.4 kcal/day), such as fried rice (Table 2.2.), than Buriram children (40.3 kcal/day). Total calorie intake was slightly higher in Bangkok children, although the difference between their intake and that of children in Buriram was not statistically significant.

We then estimated quantities of each nutrient consumed in individuals using the food-and-nutrient database in Thailand. A PCA using the log-ratio transformed nutrient matrix data showed a significant difference of dietary habits between children in Bangkok and Buriram ($p < 0.001$ in PERMANOVA analysis) (figure 2.2.). Indeed, children in Bangkok consumed significantly more fat and sugar and less beta-carotene than children in Buriram (Figures 2.1C, D). The fat consumption ratio among Bangkok children was statistically higher than among Buriram children (35.9 ± 5.9% vs. 29.7 ±

5.3%, p = 0.0017 in Student's t-test). The statistical power for this comparison was 0.92 at p = 0.05 in the post hoc analysis, indicating sufficient power of this study to address the difference in the dietary habit between these two groups. The difference in the fat consumption ratio was a result of the higher intake of fried rice, cooked breads, and processed meats by Bangkok children (Table 2.2.) and the difference in the intake of beta-carotene reflects a lower rate of vegetable consumption in Bangkok children than in Buriram children. In summary, dietary habits of participating children in Bangkok were significantly modernized and westernized like consumption of processed food, whereas Buriram children had more traditional Thai dietary habits e.g. consumptions of papaya salads with fermented fish.

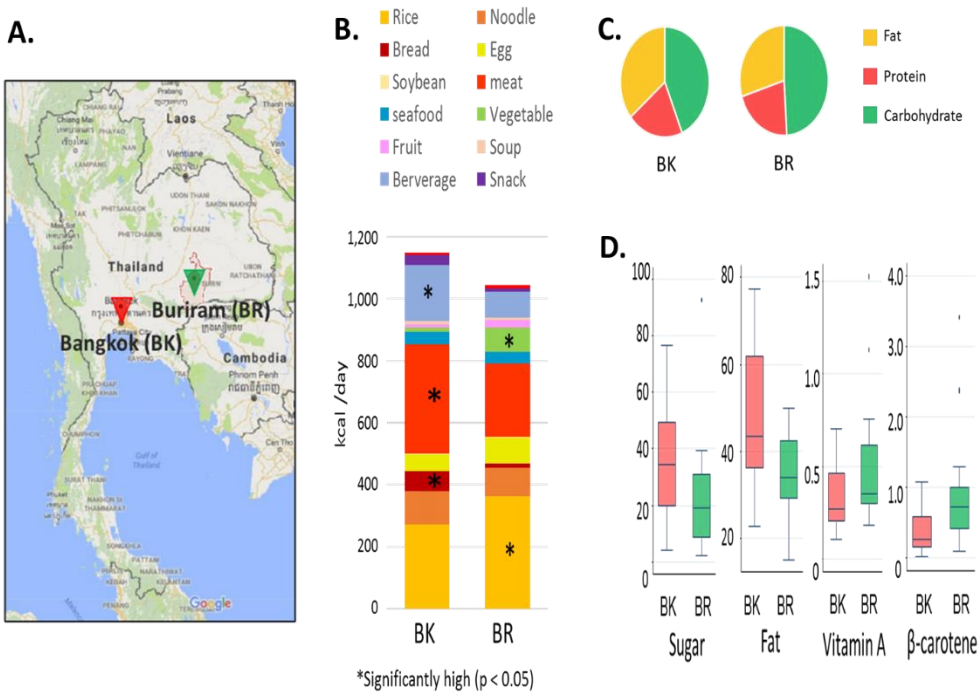

Figure 2.1. Sampling cities and diets of participants in this study. (A) Map of Bangkok and Buriram. The map was generated based on Google Maps 2017 (https://www.google.com/maps/). "BK" and "BR" were used as abbreviations. (B)

Average daily dietary intake for participants in the two cities. The contribution of each food group was estimated from the 7-day dietary record reported by participants' parent/guardian and was converted to energy units (kcal) according to databases of food energy and nutrition. Asterisks indicate statistically significant differences ($p < 0.05$) by Wilcoxon rank-sum test. (C) The energy ratio of macronutrients consumed daily in BK and BR children. (D) The nutrients in foods consumed daily showing statistically significant differences between BK and BR children ($p < 0.05$ by Wilcoxon rank-sum test). The box plots show the smallest and largest values, 25 and 75% quartiles, medians, and outliers [12].

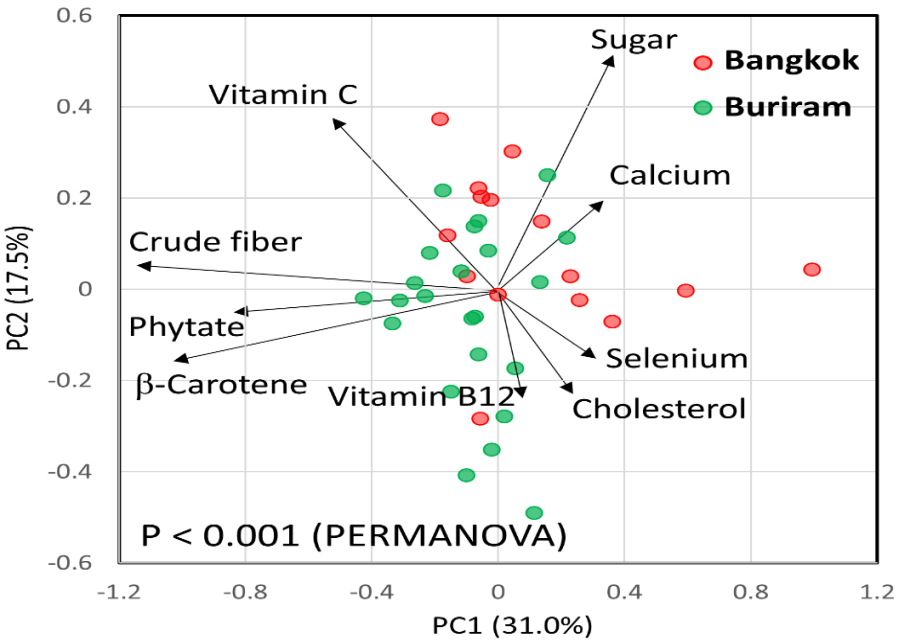

Figure 2.2. PCA of nutrient consumption of Bangkok and Buriram children. Based on 7-day food consumption day, consumed amount of each nutrient in individuals was calculated, log-ratio transformed, and applied to the PCA. Significance of difference was tested by PERMANOVA [12].

Table 2.2. Energy ratio consumed by Bangkok and Buriram children

	Bangkok (BK)	**Buriram (BR)**	**BK vs BR**
Rice form consumed	**Energy (kcal/day)**	**Energy (kcal/day)**	**p**
Steamed rice	140.8 ± 77.4	207.1 ± 93.1	1.65.E-02
Rice porridge	24.9 ± 27.3	13.6 ± 28.6	5.41.E-02
Steamed Glutinous rice	13.1 ± 20.7	101.4 ± 115.9	5.13.E-03
Single Dish (Fried Rice)	94.4 ± 89.1	40.5 ± 51.5	6.49.E-02

Energy ratio contributed by rice cooked in different forms and consumed daily in Bangkok and Buriram children. The p-value by Wilcoxon rank-sum test [12].

2.5. Discussion

Buriram children more or less retained Isan traditional dietary habits, which include foods low in fat and high in vegetables, whereas Bangkok children consumed more sugar and fat, reflecting the urbanization of their dietary culture. The average total fat intake in Bangkok children corresponded to more than 35% of total energy intake, the upper level recommended for children by the Food and Agriculture Organization. [9] The dietary records in this study indicate that the higher amount of fat intake in Bangkok children depends on cooking style more than raw materials: for example, many fried meals were served to Bangkok children. In summary, dietary habits of participating children in Bangkok was significantly modernized and westernized, whereas Buriram children had more traditional Thai dietary habits.

2.6. References

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Chapter 3

Study on the link between diet and microbial communities in children in Thailand

This chapter corresponds to a part of the original paper **(Kisuse Juma, La-Ongkham, O.; Nakphaichit, M.; Therdtatha, P.; Momoda, R.; Tanaka, M.; Fukuda, S.; Popluechai, S.; Kespechara, K.; Sonomoto, K.; Lee,YK; Nitisinprasert S and Jiro Nakayama,** Urban Diets Linked to Gut Microbiome and Metabolome Alterations in Children: A Comparative Cross Sectional Study in Thailand**)** published in Journal of Frontiers in Microbiology. 9: 1345, 2018.

3.1. Abstract

The urbanized foods may have an adverse impact on the balance of their intestinal microbial ecosystem. The observational study was conducted to compare the gut microbiota of urban (n = 17) and rural (n = 28) school-aged children in Thailand in association with their dietary habits. Sequencing of 16S rRNA genes amplified from stool samples showed that children in Bangkok have fewer *Clostridiales* and more *Bacteroidales* and *Selenomonadales* compared to children in Buriram and bacterial diversity is significantly less in Bangkok children than in Buriram children. Moreover, Bangkok children were more highly colonized by family *Porphyromonadaceae* and genera *Bacteroides* and *Bifidobacterium* than Burirum children, which coincides with the contrast between children in Italy and rural Africa. This suggests a niche established in the gut environment of urban children, which may have an impact on their intestinal environment health.

3.2. Introduction

The gastro-intestinal tract (GIT) microbiota plays an important role in the host health while changing its amount and composition under the influence of diet [1]–[3]. The diet abundant in carbohydrates, protein sources, fruits, and vegetables influenced a dominant *Firmicutes* and *Bacteroidetes* [4]. Thailand children harbor two types of enterotypes, namely *Prevotella*-type and *Bacteroides*-type, which differ by the city of residence [5]. The fact that Thai vegetarians and non-vegetarian were mainly colonized by *Prevotella*-type and *Bacteroides*-type suggests that *Prevotella*-type favours plant-based diet [6].

Thai dietary habits are reported to have changed indefinitely from consuming whole grains, fibers, rich foods, low fats to more refined carbohydrates, i.e., sugar, animal proteins, etc. This transition is influenced more by trade liberalization and globalization (Westernization) [7]. These changes lead to over nutrition consumptions accompanied by an increasing prevalence of diet-related diseases like obese, and diabetes for children and adults of Thailand [8], [9]. The metabolic health disorders are also known to be associated with alteration of gut microbiota, both richness and diversity [10]–[13].

The previous chapter has described the existence of a difference in dietary nutritional intake between urban and rural Thailand. The dietary change was comparable to those of children living in Leyte island of Philippines, which associated with gut microbiota alteration toward those of western countries [14].

This chapter aims to study further the effects of westernization of diet by assessing/investigating the gut microbiota of urban (Bangkok) and rural (Buriram) children in association with the difference of their dietary habits [2], [15].

3.3. Material and method

3.3.1. Study population characteristics

All samples used in this chapter were collected from same children as described in 2.3.1.

3.3.2. Stool sample collection for 16S rRNA and processing

Participant's collected fresh fecal samples defecated on the trailing paper into a 76 × 20-mm sterile container with 2 mL of RNAlater (Ambion, Inc., Austin, TX, United States) for microbial genomic extraction. This was collected from the three points to make a total of 0.3 – 0.5g for 16S rRNA gene analysis. The samples were immediately placed on a pre-frozen ice pack in a Styrofoam and transferred to the laboratory within 6 hours. The samples then stored in -20°C freezer until DNA extraction.

3.3.3. DNA extraction

Bacteria DNA was extracted by the bead beating method and purified as previously described [16] with modification in centrifugation speed adjusting the volume of sample and solvents used. In brief, the stool sample was diluted 10-fold with RNAlater and homogenized. Then, 200 µl of the fecal sample diluents mixed with 1 ml PBS and vortexed. After centrifugation at 20,000 × g for 5 min at 4°C, the supernatant was removed and washed twice with 1 ml of PBS buffer to remove PCR inhibitors including lipids, bile salt, hematin, calcium ions, and urea [29]. The supernatant was discarded, and

the pellet was stored at –30°C until use. 300mg of glass beads (diameter, 0.1 mm) (TOMY SEIKO Co., Ltd., Tokyo, Japan), 300 µl of Tris-SDS solution and 500 µl of TE buffer-saturated phenol were added to a thawed sample, and then shaken vigorously using a FastPrep FP120 (Bio 101) at a speed of 5.0 m/sec for 30s. 400µl of phenol/chloroform/isoamyl alcohol (25:24:1; v/v) was added to 400 µl of supernatant and shaken vigorously by using FastPrep PF120 at a speed of 4.0 m/sec for 45 s. After centrifugation at 20,000 × g for 5 min at 4°C, 250 µl of supernatant mixed with 25 µl of 3 M sodium acetate (pH 5.2). After being kept for 3 min on ice, 300 µl of ice-cold 100% isopropanol was added and centrifuged at 20,000 × g for 5 min at 4ºC. The pellet of DNA was washed in 500 µl of ice-cold 70% ethanol and air-dried before suspension in 1 ml of TE buffer (pH 8.0) and stored at –30°C until use.

3.3.4. 16S rRNA gene amplicon sequencing

The variable region, V1–V2, of the 16S rRNA gene, was amplified from the fecal genomic DNA (1 ng) using TaKaRa Ex Taq HS (Takara Bio, Shiga, Japan) and universal primers, Tru 27F (5′-CGC TCT TCC GAT CTC TGA GRG TTT GAT YMT GGC TCA G-3′) and Tru 354R (5′-TGC TCT TCC GAT CTG ACC TGC CTC CCG TAG GAG T-3′). The amplified products were then used as templates for a second PCR for further amplification with barcode-tag primers. The second-PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, United States) according to the manufacturer's protocol. The amplified DNA was quantified using a PicoGreen dsDNA Assay Kit (Life Technologies, Eugene, OR, United States) per the manufacturer's protocol. In a total of 20ng an equal amount of DNA were mixed from

each sample and was then purified by electrophoresis in a 2% (wt/vol) agarose gel, followed by extraction from the gel using a FastGene Gel/PCR Extraction Kit (Nippon Genetics Co., Ltd., Tokyo, Japan). The purified DNA was applied to paired-end sequencing using Illumina MiSeq v3 chemistry (Illumina Inc., San Diego, CA, United States).

3.3.5. Processing of 16S rRNA gene sequences

The obtained sequences were processed using the Uparse pipeline in Usearch v9.2 [17]. The pairs of raw sequence reads were merged using the fastq merge pairs script with mismatch windows up to 25 bases. High-quality sequences were selected from the merged sequences using the fastq_filter script with an expected error score lower than 1.0. Then PCR chimera-like sequences were removed by employing the Uchime algorithm. The high-quality sequences obtained were clustered using the cluster_otus script, resulting in 663 non-singleton operational taxonomic units (OTUs). The taxonomy of each OUT was assigned using the SINTAX command [18], with RDP training set v16 and a cut-off value of 0.8. The raw merged sequences before quality filtering were mapped to OTUs with identities higher than 0.97, using the usearch_global script, and the number of reads per sample assigned to each OTU counted. Eventually, 11020 ± 3948 reads per sample were assigned. The values of good's coverage for the samples were 99.23 ± 0.18% (minimum = 98.83%), indicating sufficient sequencing depth for the microbiome investigation in this study.

3.3.6. Statistical analysis for the fecal bacterial community

To test the statistical significance of differences in the fecal bacterial community structure between Bangkok and Buriram children, pairwise weighted UniFrac distance was calculated by using the beta_diversity.py command in QIIME version 1.9.1 [19] and was subjected to PERMANOVA test using the PERMANOVA function in the micropower R-package [20]. The taxonomic composition of each sample was determined, using the summarize_taxa_througy_plots.py command in QIIME version 1.9.1. [19]. The Wilcoxon rank-sum test was conducted to assess the statistical significance of variations in abundance of each bacterial community in Bangkok and Buriram samples. To determine the statistical significance of differences between the five metabolotype groups, a pairwise Wilcoxon rank-sum test was performed in R ver. 3.3.2. To examine the confounding effects of age and gender, we performed the multivariate regression analysis in Stata SE12.0 (Stata Corporation, College Station, TX, United States), for the taxonomy composition using age and city, or gender and city as the independent variables.

3.4. Results

3.4.1. Composition of gut bacteria community of Bangkok and Buriram children

There were high abundances of *Bacteroidaceae* in Bangkok children while Buriram children had high abundances of *Lachnospiraceae* and *Peptostreptococcaceae* families (Fig. 3.1.A). More than half of over 100 total OTUs found to belong to Class clostridia, especially of *Lachnospiraceae* and *Ruminococcaceae* families; meanwhile, less than 20 OTUs characterized belongs to the two dominant families, *Prevotellaceae* and *Bacteroidaceae* respectively. Furthermore, Buriram samples showed a significantly higher number of OTUs of the subdominant family, *Peptostreptococcaceae*, than Bangkok samples. (Fig. 3.1.B)

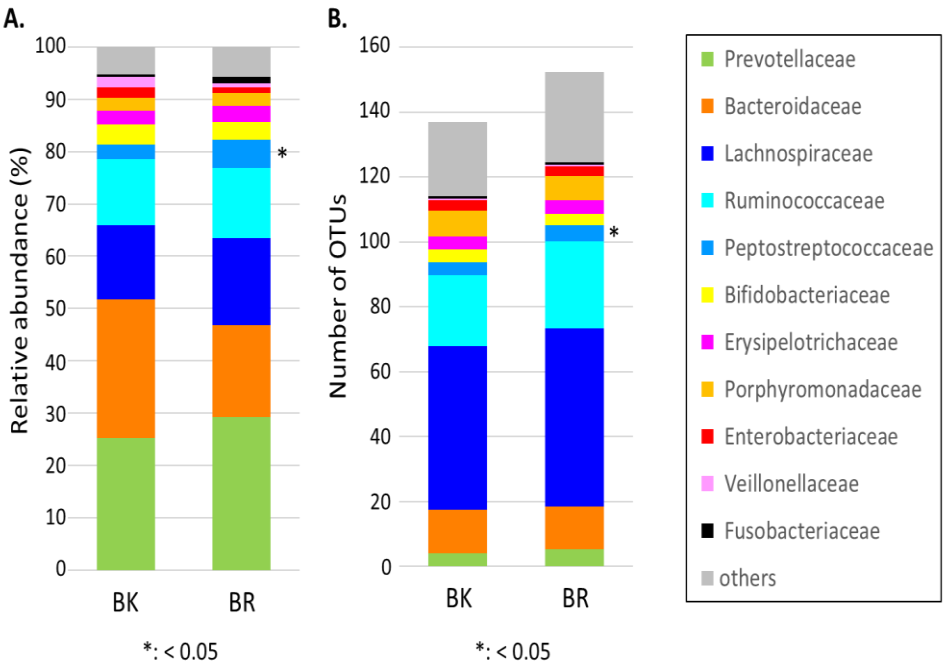


Figure 3.1. (A) Average relative abundance of fecal bacterial composition of Bangkok and Buriram children (B) The average number of taxonomic units annotated to each bacterial family in Bangkok and Buriram children. The asterisk at the right side of the bar indicates the bacterial family that was significantly more abundant in BR children than BK children ($p < 0.05$, Wilcoxon rank-sum test) [30].

3.4.2. Alpha diversity of gut bacterial community of Bangkok and Buriram children

Total alpha diversity tended to be higher in Buriram samples than in Bangkok samples, as indicated by Chao1 ($p = 0.042$) and ACE ($p = 0.047$) species richness estimates. (Figure 3.2)

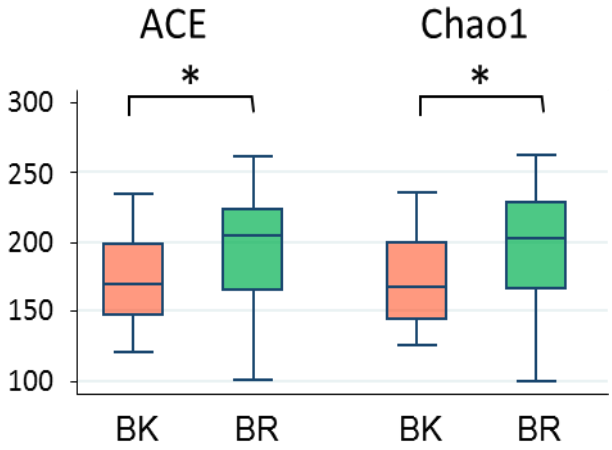


Figure 3.2. Alpha-diversity indices on fecal microbiota of children in Bangkok and Buriram which were compared by the Wilcoxon rank-sum test. Asterisks indicate $p < 0.05$ [30].

3.4.3. Difference in gut bacterial community between children in Bangkok and Buriram

The variance of microbial community was profiled by using pairwise weighted UniFrac distances calculated based on the OTU tables of Bangkok and Buriram samples. The weighted UniFrac distances between samples showed no statistically significant differences in the microbial community structures between Bangkok and Buriram children. We further performed PCA using bacterial composition data at the family level. As shown in Fig. 3.3. A., the three enterotype-like variations were observed, although they were not entirely discrete. At the PC1-positive region, it was defined by a high abundance of *Bacteroidaceae* whereas, at the PC1-negative region, it was characterized with a high abundance of *Prevotellaceae* and the third one, localized in the PC2-negative region, as defined by a high abundance of the combination of *Bifidobacteriaceae*, *Lachnospiraceae*, *Ruminococcaceae*, and *Peptostreptococcaceae* (Fig. 3.3.A). Bangkok and Buriram samples appeared to be oriented in PC2-positive and -negative directions, respectively, but there was no apparent connection between the enterotype-like cluster and the cities of residence. (Fig. 3.3.B)

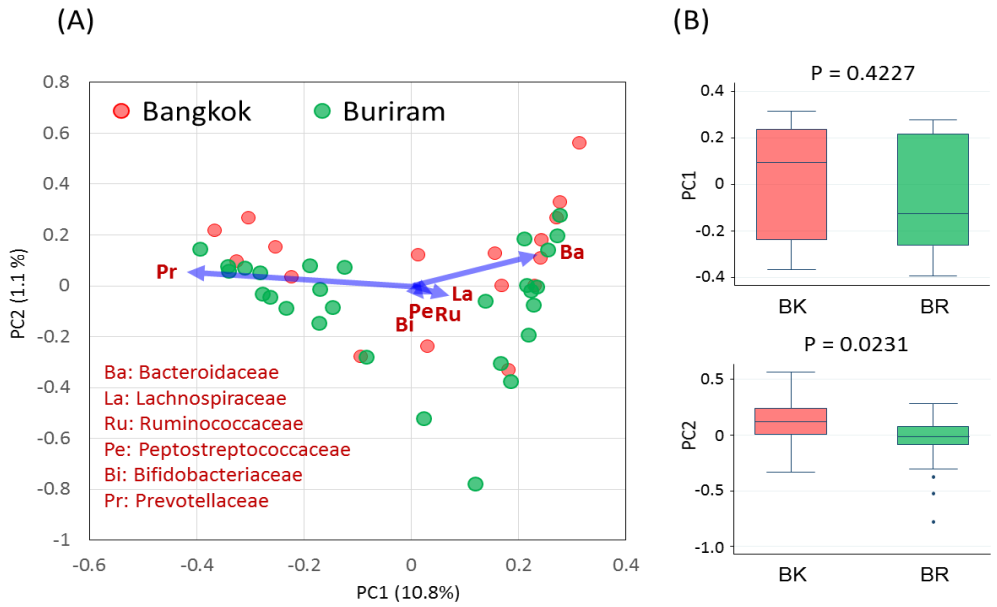
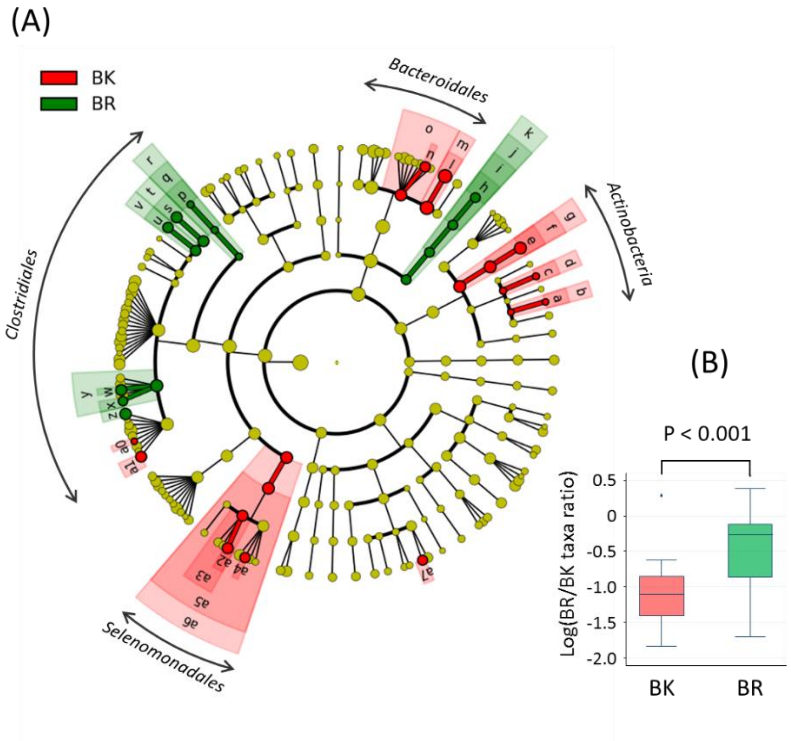


Figure 3.3. Principal component analysis (PCA) of fecal bacterial community profiles (family level) of children in Bangkok and Buriram. **(A)** PCA plot of 17 Bangkok and 28 Buriram children. **(B)** PC1 (upper) and PC2 (lower) sample distributions. The probability value (*p*) was determined by Wilcoxon rank-sum test [30].

3.4.4. Linear discriminant analysis effect size (LEfSe) analysis

A LEfSe study was conducted using phylum to genus-level data to identify more specific variations in the fecal bacterial compositions among children in the two towns. As seen in Fig. 3.4.A., children from Bangkok were more highly colonized by *Actinobacteria* classes and *Selenomadales* and *Bacteroidales* orders. In contrast, children from Buriram were more heavily colonized by other taxonomic groups belonging to the order *Clostridiales*, such as families *Peptostreptococcaceae* and unclassified *Ruminococcaceae*. In Fig. 3.4.C, the relative abundance of taxonomic groups showing an LDA score greater than 10^3 (taxa indicated by bold letters in Fig. 3.4.C)

summed for the Bangkok type (*Negativicutes* + *Bacteroides* + *Porphyromonadaceae* + *Bifidobacterium*) and the Buriram type (*Peptostreptococcaceae* + unclassified *Ruminococcaceae*), and the ratio of the sum of the Buriram type to the sum of the Bangkok type calculated for each child (henceforth, the ratio is called the BR/BK taxa ratio). The BR/BK taxa ratio was significantly different between children in Bangkok and Buriram, as seen in Fig. 3.4.B ($p < 0.001$ in Wilcoxon rank-sum test). In the post-hoc review, the statistical power for this contrast was 0.88 at $p = 0.05$, suggesting adequate influence from this sample to investigate the alteration between Bangkok and Buriram children in the gut microbial population.



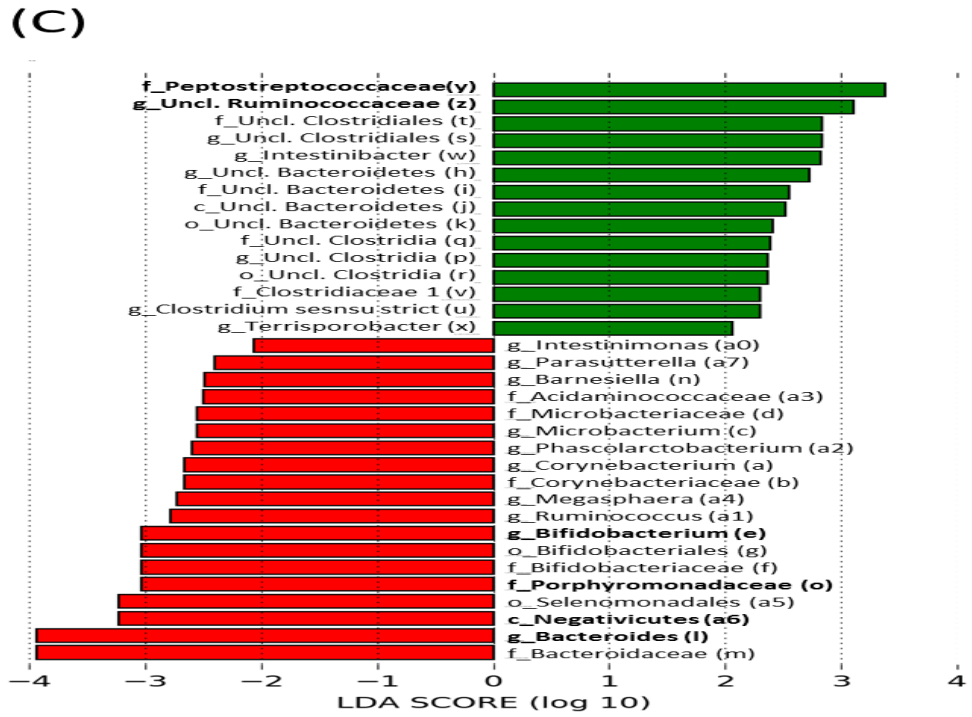


Figure 3.4. Linear discriminant analysis effect size to identify different abundant taxa between Bangkok and Buriram samples. **(A)** Cladogram showing different abundant taxa between Bangkok and Buriram (LDA score > 2.0, $p < 0.1$). **(B)** Boxplot showing distribution of log-ratio of different abundant taxa **(C)** Taxonomic groups showing LDA score > 2.0 with $p < 0.1$ [30].

3.5. Discussion

In our previous study on the gut microbiota in an Asian population, two enterotype-like clusters, one driven by *Prevotella* and the other one driven by *Bacteroides*, were found, and overrepresentation of each enterotype was observed in distinct populations, such as *Prevotella*-type microbiota in Thai vegetarians [6], and in the rural cities of Khon Kaen in Isan region of Thailand [5], and Baybay on Leyte island in the Philippines [14]. These results suggested that low-fat and vegetable-based diets promote the colonization by *Prevotella*-type microbiota. The *Prevotella*/*Bacteroides* trade-off was also found in a study comparing the gut microbiota of children from Burkina Faso consuming a rural African diet rich in dietary fiber and Italian children consuming a modern Western diet [2]. Further, recent studies have observed the *Prevotella*/*Bacteroides* trade-off within a country [14], [21], suggesting that the enterotype shift is ongoing in developing countries. Unexpectedly, however, we did not observe the enterotype shift between Bangkok and Buriram children. Preliminary data showed that the enterotypes were not consistent in this Thai cohort across samplings with a 1-month interval, while most Japanese children were consistently colonized by a *Bacteroides*-type enterotype. This could suggest that the gut microbiota of Thai children is now being influenced by modern diets and are in a state of transition.

Rather than the enterotypes reflected in PC1 of the PCA, the city of residence correlated somewhat with PC2, which mainly reflected the *Firmicutes*-to-*Bacteroidetes* (F/B) ratio. The F/B ratio is known to be positively correlated with high-fat diet-induced obesity [22]–[25]. In this study, however, Bangkok children, who tended to consume a high-fat diet and to be overweight, showed a lower F/B ratio. This agrees with a line of studies suggesting that species, community, or functional level studies are required to

understand the causation between diets and microbiota [26]–[28]. Indeed, some families or genera, such as *Bacteroides*, *Bifidobacterium*, and *Porphyromonadaceae*, are commonly higher in children in Italy [21] as well as children in Bangkok, suggesting a niche established in the gut environment of urban children.

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Chapter 4

Metabolomic alteration of gut microbiota of Thai children in association with loss of traditional diets

This chapter correspond to a part of the original paper **(Kisuse Juma, La-Ongkham, O.; Nakphaichit, M.; Therdtatha, P.; Momoda, R.; Tanaka, M.; Fukuda, S.; Popluechai, S.; Kespechara, K.; Sonomoto, K.; Lee,YK; Nitisinprasert S and Jiro Nakayama,** Urban Diets Linked to Gut Microbiome and Metabolome Alterations in Children: A Comparative Cross Sectional Study in Thailand**)** published in Journal of Frontiers in Microbiology. 9: 1345, 2018.

4.1. Abstract

Food globalization has altered the Thai dietary style, which is supposed to affect their gut microbial ecosystem. In this chapter, to address this point, the fecal metabolites were profiled by using fecal samples of school-age children living in an urban city, Bangkok (n = 17), and rural city, Buriram (n = 28), in Thailand. Both butyrate and propionate were more abundant in Buriram children than in Bangkok children. The clustering yielded five metabolotypes (MTs), each characterized by high concentrations of short and middle chain fatty acids (MT1, n = 17), amino acids (MT2, n = 7), arginine (MT3, n = 6), low level of metabolites (MT4, n = 4), and amino acids and amines (MT5, n = 8).MT1 and MT4 consisted mainly of samples from Buriram, and MT2 and MT3 consisted mainly of samples from Bangkok, while MT5 contained three samples from Bangkok and five samples from Buriram. MT1 and MT2 were accompanied by the typical microbiome and diet consumption of Buriram and Bangkok children, respectively. Predicted metagenomics using the 16S rRNA profiles revealed an under-representation of eight genes involved in butyrate biosynthesis pathways in MT2, including both glutamate and pyruvate pathways. Taken together, this study shows the benefit of high-vegetable Thai traditional diets on gut microbiota and suggests that high-fat and less-vegetable urban dietary habits alter gut microbial communities in Thai children, which resulted in the reduction of colonic short-chain fatty acid fermentation.

4.2. Introduction

As the end product of dietary metabolism, a variety of fecal metabolites are produced by the gut microbial community, therein short-chain fatty acids (SCFA) being major metabolites which play crucial roles in health and diseases of human being [1]–[7].

Diets shape the functional activity of the gut microbiota and thereafter profile of the fecal metabolome provides an insight of the health of individuals. [8] There is growing concern over urbanization that causes changes in the dietary richness of Thailand, which are supposed to alter the composition and activity of gut microbial community, resulting in the loss of production of potentially beneficial metabolites. Increase consumptions of fats, proteins, and refined sugars may halt the goodness of the Thai traditional diet [8], [9].

The aim of this chapter is to compare fecal metabolite composition between rural and urban areas in Thailand and to address any potential variations that may occur in relation to change of health status as well as dietary urbanization in Thai children.

4.3. Material and method

4.3.1. Study population characteristics

All samples used in this chapter were collected from same children as described in 2.3.1

4.3.2. Stool sample collection and processing for metabolome analysis

All participants collected samples for metabolome analysis from fresh defecated feces on the trailing paper of about one big spatula (> 1g) into 55 X 44 mm, Sarstedt. The samples were immediately placed on a pre-frozen ice pack in a Styrofoam and transferred to the laboratory within 6 hours. It was then transferred to -80°C freezer and lyophilized and then stored back to -80°C freezer until metabolite extraction.

4.3.3. Capillary electrophoresis time of flight mass spectrometry (CE-TOF MS) measurement

The author analyzed stool metabolites as previously described [10], [11]. Briefly, fecal metabolites were extracted by vigorous shaking with methanol containing 20 μM each of methionine sulfone, D-camphol-10-sulfonic acid, and 2-(*N*-morpholino) ethane-sulfonic acid as the internal standards, and were then cleaned by chloroform and water extraction and ultrafiltration using Ultra free-MC (UFC3 LCC NB, 5,000 NMWL, black label). The purified fractions were analyzed using a CE-TOF MS system consisting of an Agilent CE capillary electrophoresis system (Agilent Technology, Palo Alto, CA,

United States), Agilent G32500AA LC/MSD TOF system (Agilent Technologies), Agilent 1100 series binary high-performance liquid chromatography pump, G1603A Agilent CE-MS adapter, and G1607A Agilent CE-ESI-MS sprayer kit. The metabolites were quantified for the study using a standard curve derived from standard samples. Concentrations below the detection limit were substituted with zero, and metabolites whose levels were below the detection limit in all of the samples were excluded.

Pearson's association between samples was determined using composition of the whole metabolite and subjected to hierarchical clustering. In order to find metabolites abundant or depleted in a specific metabolotype group, the concentration of each metabolite underwent a Wilcoxon rank-sum test between one group and all other groups combined. A pairwise Wilcoxon rank-sum test with or without Bonferroni adjustment in R v. 3.3.2 was performed to determine the statistical significance differences in the amounts of certain metabolites in the metabolotype groups. Pearson's correlation between each metabolite and the bacterial abundance was then determined by Pearson's correlation coefficient test in Stata SE12. To examine the confounding effects of age and gender, the author conducted a multivariate regression analysis in Stata SE12 using butyrate or propionate concentrations as dependent variables and age and city or gender and city as independent variables.

4.3.4. Linear discriminant analysis effect size (LEfSe)

The LEfSe [12] was calculated using the online version of Galaxy (http://huttenhower.sph.harvard.edu/galaxy/root, version 1.0.0). For OTUs with an average abundance in all samples that was greater than 0.1%, abundances were

normalized to the sum of the values per sample in 1 million and then subjected to linear discriminant analysis (LDA). The LDA was performed using a one-against-all strategy, and OTUs showing a score higher than 2.0 were selected.

4.4. Results

4.4.1. Fecal SCFAs in Bangkok and Buriram children

The fecal metabolic analysis were quantified using a CE-TOFMS and found that butyrate and propionate concentrations differed significantly between children in both cities (Figure 4.1.). In addition, butyrate concentrations were positively correlated with BR/BK taxa ratios of sufficient statistical significance and power (Figure 4.2.). The findings revealed that the city of residence was strongly correlated with both the butyrate level and the propionate level even after the adjustment, indicating that there was no confounding impact on age and gender in the SCFA level study.

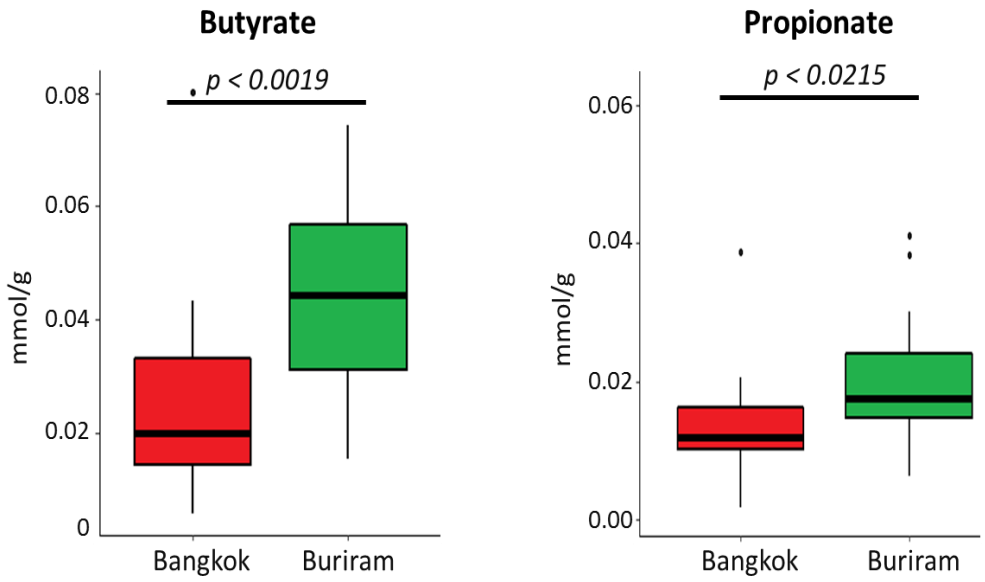

Figure 4.1. Comparison of the concentrations of fecal SCFAs between children in Bangkok and Buriram. P-value by Wilcoxon sum-rank test.

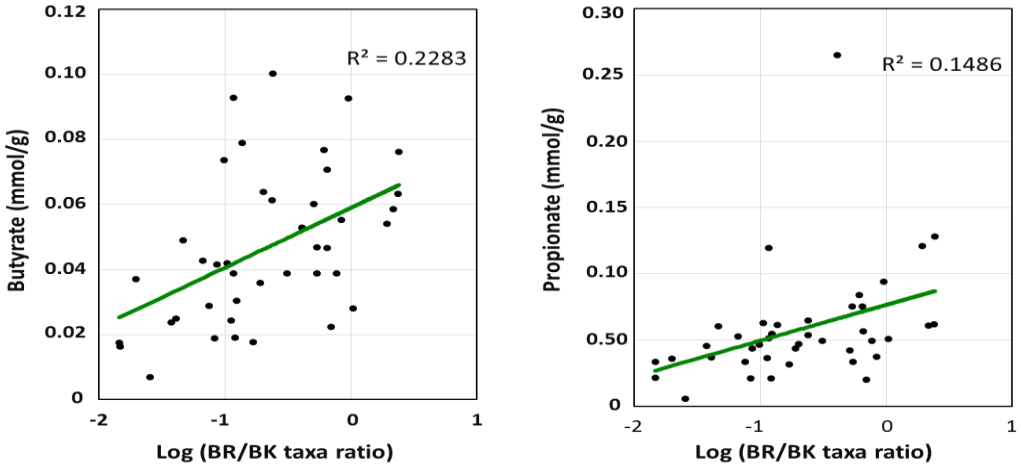

Figure 4.2. A correlation between SCFA and ratio of bacterial composition of the city of residence. All the probability value (*p*) was calculated by Wilcoxon rank-sum test [27].

4.4.2. Metabolotypes found in the fecal metabolite profiles of children in Bangkok and Buriram

Using fecal metabolome data, a cluster analysis was performed and resulted in five (5) clusters (Figure 4.3. A.). Metabolites characterizing fecal metabolotypes were selected, and their abundances are shown in a heat map (Figure 4.3.A.) and box plots (Figure 4.3. B.). Notably, these clusters were characterized by high abundance of SCFAs in metabolotype-1 (MT1), amino acids in metabolotype-2 (MT2), arginine in metabolotype-3 (MT3), and amines in metabolotype-5 (MT5), whereas metabolotype-4 (MT4) totally lacked in metabolites.

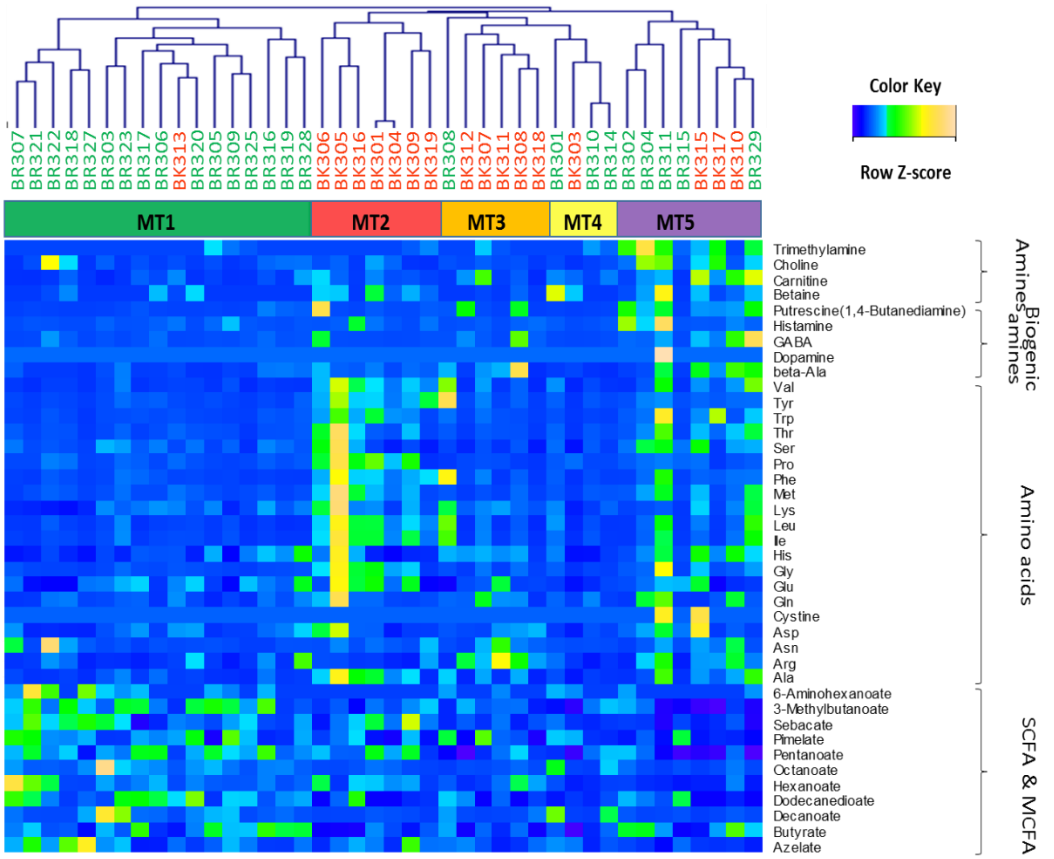

Figure 4.3. Five metabolotypes based on the measurement of 214 metabolites in stool samples from children in Bangkok and Buriram. Metabolites characterizing fecal metabolotypes were selected, and their abundances are shown in a heat map **(A)** and box plots **(B). (A).** A hierarchical clustering dendrogram was generated based on Pearson's correlation determined based on the abundance of whole metabolites. Samples coloured by subjects' city of residence and clusters coloured by metabolotype were shown between the dendrogram and heat map. The abundance of each metabolite was converted to a *Z*-score across all samples and displayed in the heat map according to the above colour key [27].

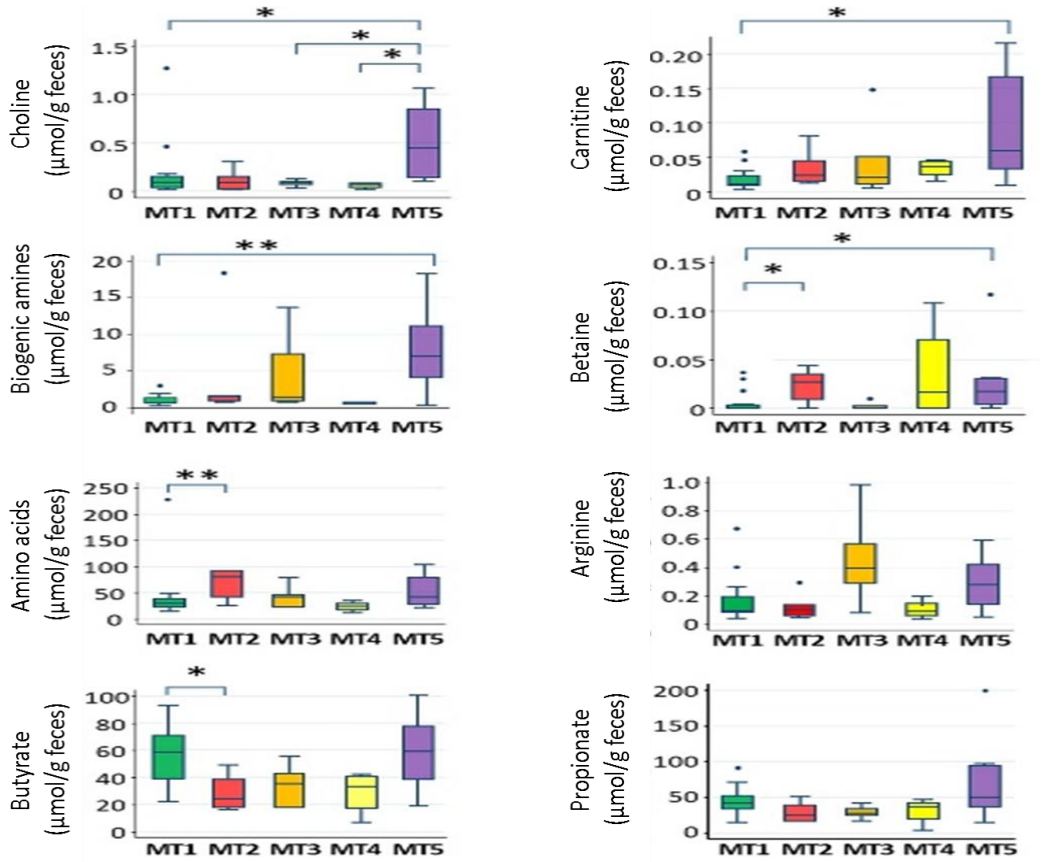

Figure 4.3. B. Single and double asterisks indicate $p < 0.1$ and $p < 0.05$, respectively, in a pairwise Wilcoxon rank-sum test with Bonferroni adjustment. "Biogenic amines" represent the sum of beta-alanine, dopamine, gamma-aminobutyric acid, histamine, phenethylamine, putrescine, and tyramine. "Amino acids" represent the sum of 20 standard proteinogenic amino acids [27].

4.4.3. The bacterial compositions of these five metabolotype groups were compared by LEfSe analysis

Except for one sample from a child in Bangkok, all the MT1 samples were derived from Buriram children. MT1 samples contained middle chain fatty acids (MCFAs) such as capric and caproic acids, in addition to two SCFAs measured. The

microbiota of MT1 samples was characterized by a high BR/BK taxa ratio and high alpha diversity (Figures 4.4. B and C), as observed in Buriram children. On the other hand, MT2 samples were characterized by high concentrations of proteinogenic amino acids (Figure 4.3. A). All of the MT2 samples were derived from Bangkok children and showed a low BR/BK taxa ratio (Figure 4.4. B). MT3 was also mainly derived from Bangkok children. The bacterial composition of this group was characterized by a high abundance of *Parabacteroides*, whereas the BR/BK taxa ratio was intermediate between those of MT1 and MT2. Taken together, MT1 represents the high-SCFA-producing microbiota of Buriram children, and MT2 represents the low-SCFA producing microbiota of Bangkok children.

MT4 was characterized by the overall low level of fecal metabolites. This group comprised three samples from Buriram and one from Bangkok. The microbiota of MT4 was characterized by a high abundance of *Fusobacterium* and *Desulfovibrionaceae* and low alpha diversity (Figure 4.4. C).

MT5 comprised five Buriram and three Bangkok samples. This type was characterized by high concentrations of choline, betaine, and carnitine, which are generally abundant in red meat and dairy products, and a series of biogenic amines such as gamma-aminobutyric acid (GABA) and putrescine. A number of previous studies have shown that these meat-derived amines are converted to trimethylamine by gut bacteria and further metabolized by a liver enzyme to trimethylamine N-oxide, which is associated with metabolic syndrome, fatty liver disease, and cancer [13]. In the stool samples from Thai children, trimethylamine concentrations were positively correlated with amine levels and were significantly more abundant in participants in the MT5 group. The microbiota

associated with MT5 samples was characterized by a high abundance of the genera *Haemophilus* and *Sutterella* (Figure 4.4. A) and low alpha diversity (Figure 4.4. C).

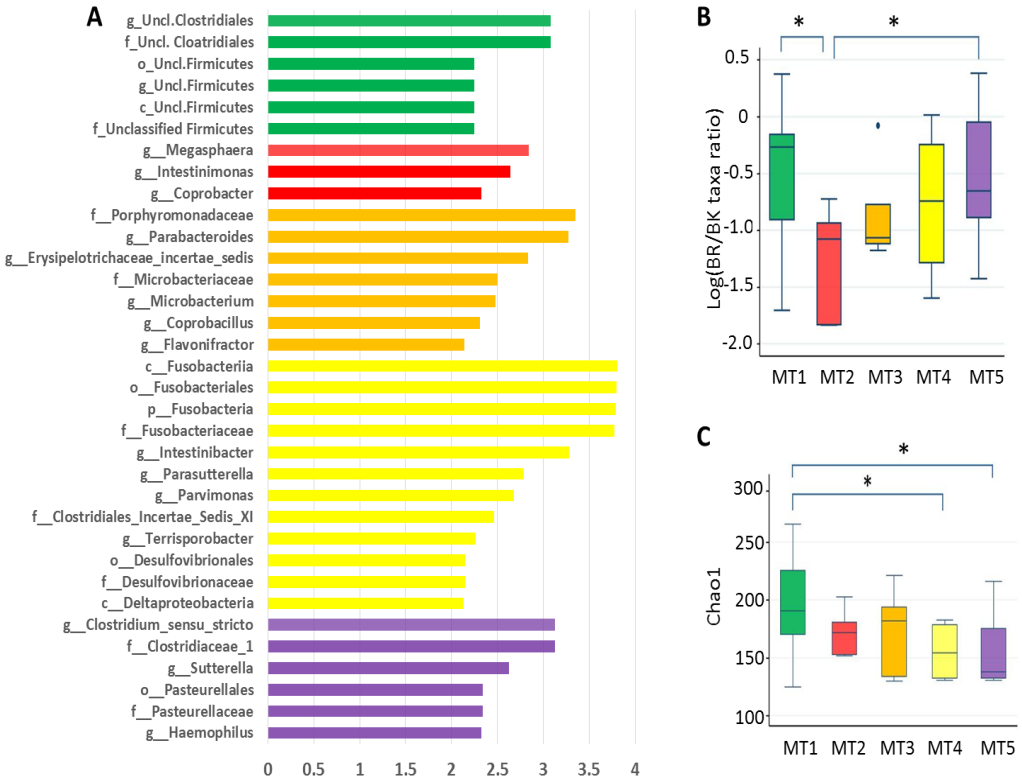

Figure 4.4. Fecal microbiota of five metabolotype groups. **(A)** Taxa overrepresented in each metabolotype group. Taxa with linear discriminant analysis scores >2.0 with $p < 0.05$ are shown. p, phylum; c, class; o, order; f, family; g, genus. **(B)** The BR/BK taxa ratio in each metabolotype group. **(C)** Chao1 index scores in each metabolotype group. Asterisks indicate $p < 0.05$ in a pairwise Wilcoxon rank-sum test without Bonferroni adjustment [27].

4.4.4. Predicted metagenomics for metabolotypes

Genes and their functions encoded by the fecal bacterial community were quantitatively predicted by PICRUSt based on 16S rRNA gene profiles. The abundance of each KEGG pathway group was compared among the metabolotypes. As a result, KEGG pathways involved in "metabolism" and some other subcategories shown to be overrepresented in the MT2 group compared with other groups, particularly MT1. Therefore, we compared the abundances of all KEGG annotated genes between groups MT1 and MT2, and genes overrepresented in the MT1 group were mapped in the KEGG pathway. The results showed that MT1 was enriched in genes involved in the metabolism of plant-related compounds, such as flavonoid, carotenoid, and limonene.

MT2 was enriched in nitrogen metabolism, including amino acid metabolism, as well as lipid and energy metabolism, whereas MT1 was enriched in ketone body metabolism and phosphotransferase system as well as butanoate metabolism. Notably, 8 genes were overrepresented in butyrate biosynthesis pathways in MT1, including paths from glutamate as well as pyruvate (Figure 4.5.). Those genes involved in the paths from glutamate are mainly encoded by bacteria in the genus *Romboutsia*, family *Peptostreptococcaceae*, whereas those in the paths from pyruvate are encoded by various genera, although *Romboutsia* is one of the significant contributors. Further, 19 genes involved in the phosphotransferase system, including cellobiose phosphotransferase, were overrepresented in MT1, suggesting that higher capacity of MT1 microbiome to ferment a variety of sugars. There was no statistical difference in the total abundance of propanoate metabolism between MT1 and MT2. However, 8 genes, including propionate CoA-transferase [EC: 2.8.3.1] gene, were statistically enriched in the pathway of propanoate metabolism of the MT1 group.

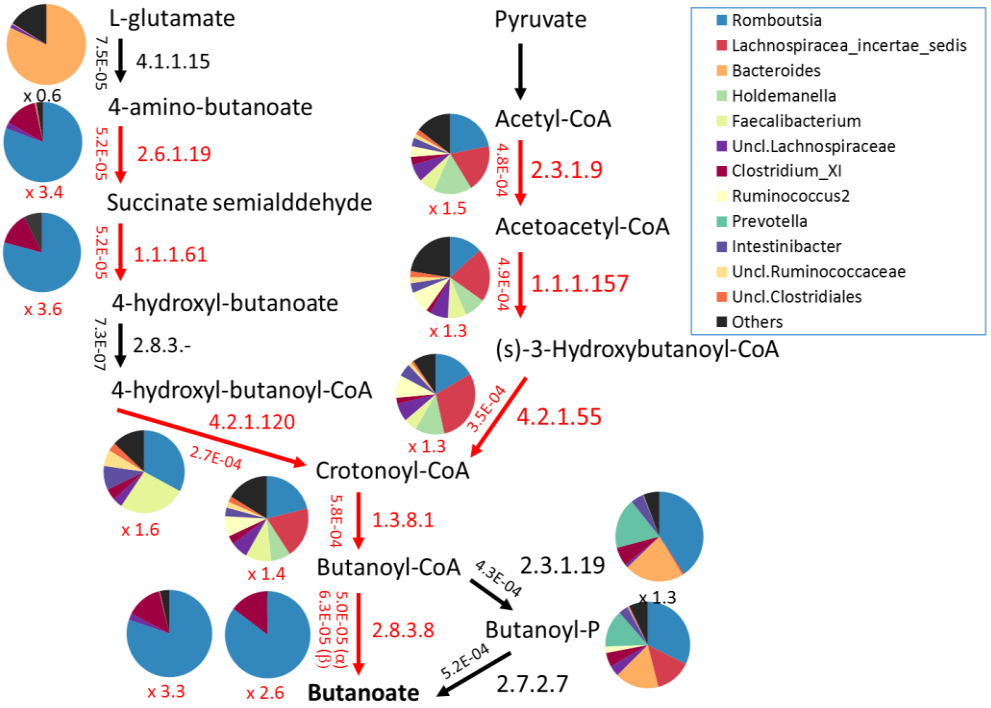

Figure 4.5. Predicted abundance of genes involved in the butyrate biosynthetic pathway. Abundances of genes were predicted by PICRUSt and are shown beside the arrow in each path represented by the EC number. Pie charts represent the contribution of bacteria genera to each path. Red arrows and letters indicate paths significantly overrepresented in Buriram samples [27].

4.4.5 Link of dietary nutrients to gut microbiome and metabolome

We examined the correlation between dietary nutrients and gut microbiome in Thai children. As a result, fat intake level showed a significant negative correlation with the BR/BK taxa ratio as shown in Figure 4.6.A. ($R2 = 0.16$, $p = 0.014$ by Pearson's correlation analysis, and Power = 0.85 in post hoc analysis), suggesting the significant

impact of high level fat consumption on the gut microbiota. Furthermore, we examined the association between dietary nutrient and fecal metabolotypes (Figure 4.6.B.). Among the macronutrients, only fat consumption showed a significant association with metabolotypes. In addition, beta-carotene intake level significantly differed among the metabolotypes groups. Children in MT2 group consumed more fat and much less beta-carotene than those in other metabolotype groups, especially MT1. Altogether, the differences in dietary habits and gut environment between two cities were reflected to the two metabolotypes, MT1 and MT2.

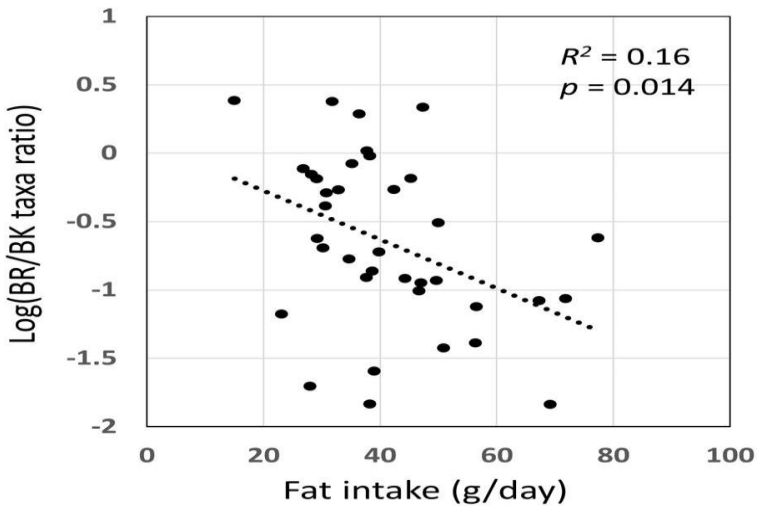

Figure 4.6. A. Correlation of fat intake level with BR/BK taxa ratio. Pearson's correlation analysis was performed between fat intake level and the BR/BK taxa ratio and a significant correlation ($p < 0.05$) was obtained [27].

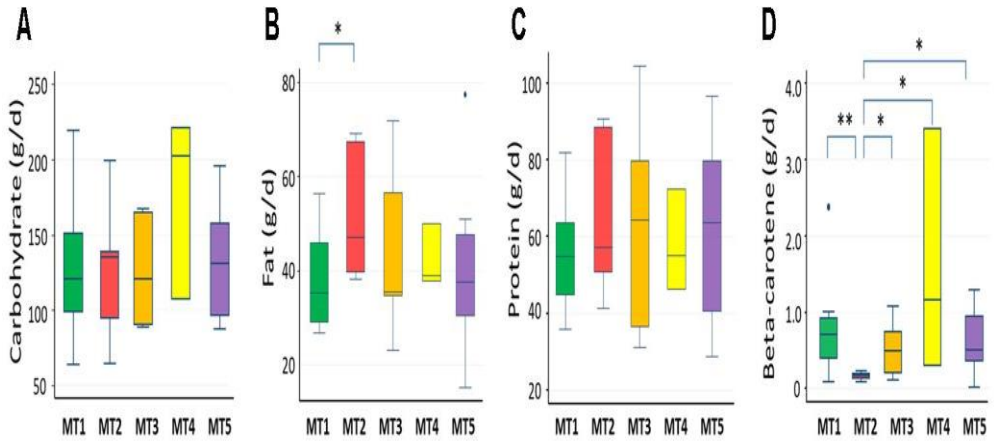

Figure 4.6.B. Levels of nutrients consumed by children in each metabolotype group. (A) Carbohydrates, (B) fats, (C) proteins, (D) beta-carotene. Double and single asterisks indicate $p < 0.05$ in pairwise Wilcoxon rank-sum tests with and without Bonferroni adjustment, respectively [27].

4.5. Discussion

Several studies have demonstrated that plant-based diets promote colonic fermentation of SCFAs and have a profound effect on host health [14]–[16]. A line of African children gut microbiota studies have commonly indicated the high level of the series of SCFAs [17], [18]. Although acetate was not measurable in our CE-TOF-MS system, the rural children in Thailand also showed a higher level of propionate and butyrate than Bangkok children. The line of studies on Africans and Asians demonstrated that their rural microbiomes have a higher capacity to accept a variety of sugars, including overrepresentations of phosphotransferase genes and carbohydrate-active enzyme genes [19]. These results suggested that the human gut microbiome primarily evolved to utilize a wide range of carbon sources and was then narrowed down under the excessive intakes of simple sugars.

The cluster analysis based on the top-down metabolomics data generated MT1 representative of Buriram metabolome. Samples in the MT1 group were characterized by higher concentrations of SCFAs and higher alpha diversity. It is interesting that the MT1 samples also showed higher concentrations of MCFAs. Microbial production of MCFAs is not common in the human gastrointestinal tract. They may be because of the consumption of coconut milk or palm oil, which are common ingredients in Thai food. However, we could not find any correlation between MCFAs in stool samples and the dietary records of MT1 children.

MT2, characterized by a high abundance of amino acids and low level of SCFAs, showed a lower BR/BK taxa ratio representative of the microbiota in Bangkok children. The dietary habits of MT2 children were characterized by high-fat and remarkably low-vegetable consumption. Although there is no direct evidence, this

suggests that their urbanized dietary habit altered the gut microbial communities of Bangkok children and reduced the microbial production of SCFAs. Whereas, SCFAs in the intestine benefit the host, the overrepresentation of amino acids in the gut has been found to associated with gut dysbiosis in patients with Crohn's disease (CD) [20]. This paper reported a 15N flux study in mice that showed bacterial urease releases ammonia by hydrolysis of host urea and allows the transfer of host-derived nitrogen to the gut microbiota as a source of amino acid biosynthesis.

Interestingly, the predicted metagenomics showed that *Peptostreptococcaceae*, which was overrepresented in Buriram children, was involved in the biosynthesis pathway from glutamate to butyrate. Although *Peptostreptococcaceae* is not a common butyrate producer such as *Faecalibacterium* or *Roseburia* [21], some reports are showing that this taxonomic group can produce SCFAs from amino acids [22], [23]. The path from amino acids to butyrate may play an essential role in maximizing SCFA production from limited carbon sources in the colon.

MT4, characterized by the overall low level of fecal metabolites, had a high abundance of Fusobacterium and *Desulfovibrionaceae*, which are known to be overrepresented in patients with colon cancer and ulcerative colitis and to be involved in inflammation [24], [25]. Notably, the BMIs of these MT4 subjects were lower than those of the other groups, except for those in the MT1 group, which might be related to their unusual gut microbiota and environments. On the other hand, MT5 was characterized as rich in metabolites, especially amines and amino acids.

Interestingly, the CD patients in the paper, as mentioned earlier, were also abundant in fecal amines, similar to patients with inflammatory bowel disease reported

elsewhere [20], [26]. Furthermore, a high abundance of Haemophilus belonging to potentially pathogenic bacteria in MT5 subjects was also common in CD patients [20].

Although numerous studies have shown, in terms of physiology as well as toxicology, the diverse activity of biogenic amines, those in the intestine should be further investigated. It should be noted that the same contrast in fecal amino acids and biogenic amines was observed in Italians and Hadza hunter-gatherers [16], suggesting that dysbiosis-like metabolomic changes are a sign of stress in gut microbial communities under the urbanization of diets.

4.6. References

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Chapter 5

In vitro study on the influence of sticky rice on GIT microbial community

5.1. Abstract

Rice is the major staple food in Thailand. In the northern and northestern regions of Thailand, sticky rice, also known as glutinous rice, called “Khao Lam” in Thai, as well as Thai jasmine rice “Khao Hom” are consumed. In this chapter, the author conducted a pH-controlled in vitro culture with fecal inoculant to investigate the effect of sticky rice on the gut microbial community in comparison with the jasmine rice. The composition of bacteria and short-chain fatty acids were monitored in this model up to 24h. In both media added jasmine and sticky rice powder, lactate sharply increased after 12h with an increase in Bifidobacterium, whereas it was not observed in the control batch suggesting Bifidobacterium growth with lactate fermentation using rice carbohydrate. On the other hand, it was found Bacteroides favoured sticky rice to jasmine rice. Hence, the promotion of Bifidobacterium is expected to benefit host health, while the health benefit of Bacteroides requires further studies.

5.2. Introduction

Traditional Thai diet is rich in vegetables, fruits, legumes, cereals and low in animal fat and proteins. It is reported to be associated with good health life and prevention of diseases [1]. Since the 13th Century, rice has been the major staple crop for both export and local consumption within Thailand [1], [2]. Rice production in Thailand mainly focuses on two major varieties of rice namely, jasmine rice and sticky rice [2], while their consumption depends on region [3], [4], [28]. Thai households consumed lesser rice per person per year in 2002 compared to 1990, which was thought to be affected by urbanization [5]. Moreover, the demand for rice in recent years has risen with low supply which is also threatened by urbanization [2], [5]. By definition jasmine rice is a white colour long-grain of indica variety with an evident aroma and contain large amount of amylose, mainly grown in Thailand, while Thai sticky rice is a short-grain of japonica variety, with a relatively large amount of amylopectin which makes it sticky. Thai sticky rice is grown mainly in the Northeast region and is an essential ingredient in Thai dessert, whereas jasmine rice is the most famous white rice in Thai [25].

Present study shows that children of Bangkok (urban) as well as Buriram (rural) had a frequency of rice consumption (Fig. 5.1 a & b) which has confirmed that rice is the main source in their diet. Buriram children highly consumed the sticky rice prepared by steaming as well as jasmine rice. This cooking method enhances colonic health formation [6] of resistant starch (RS) type 3 [7] which is later enhanced by retrogradation and promote colonic SCFA fermentation [8], [9].

Hence, the effect of sticky rice on gut microbiota and its production of SCFAs is yet to be established. Moreover, the information on the colonic fermentation of sticky rice is very limited in comparison to that of jasmine rice fermentation [10]. Hence the

author compare the influence of sticky rice on gut microbial community with that of jasmine rice findings using in vitro culture system with the inoculant of *Prevotella* or *Bifidobacterium* rich feces, respectively, and monitoring of the SCFA production and change in gut microbiota [11].

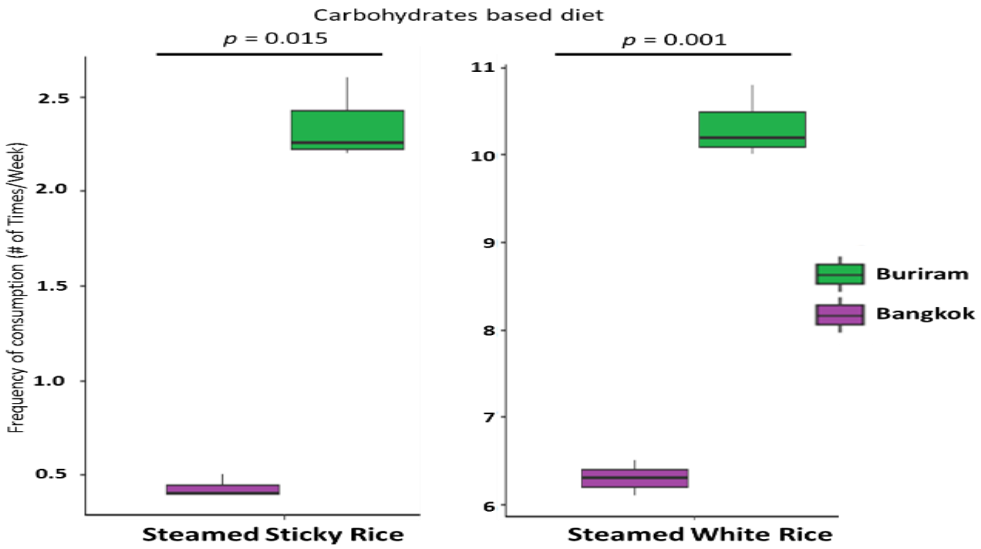

Figure. 5.1. Shows frequency of carbohydrates-based diet intake significantly frequently consumed high by Buriram children as compared to the Bangkok children. P-value calculated by Welch t.test.

Table 1.1. Statistics of 1-week shows energy gained following rice consumption in Bangkok and Buriram children.

	BK (n=15)	BR (n=27)	
	Energy (kcal/day)		t.test
Steamed rice	140 ± 77.4	209.1 ± 91.9	0.015
Rice porridge	24.9 ± 27.3	13.8 ± 28.1	0.221
Steamed glutinous rice	13.1 ± 20.7	97.6 ± 115.3	0.001

Energy gained was from the frequency of rice consumption per week. Statistical test was calculated by Welch t.test. [12].

5.3. Materials and method

5.3.1. Study design

The stool was collected from two adult participants (Table 5.2) who have no past gastrointestinal diseases, no antibiotic usage within the previous 3 weeks. Since the volunteers' gut microbiota has been previously analysed, they were chosen for the current study representing *Prevotella* (P) - and *Bifidobacterium* (B)-type respectively (figure 5.4). Fresh feces were collected into a 76 X 20-mm sterile container with 2 mL of RNAlater (Ambion, Inc., Austin, TX, United States) for 16S rRNA gene sequencing, and a 55 X 44-mm sterile container for metabolites (Short-Chain Fatty Acid - SCFA) extraction (Sarstedt, Nümbrecht, Germany) for the control followed by the in vitro fermentation. The fecal inoculum for the in vitro culture was collected using a BD CultureSwab Plus

system contains a 5-mL Amies agar gel column for preserving both aerobic and anaerobic organisms from the fecal samples.

Table 5.2 Characteristics of the fecal inoculant

	F1 (*Prevotella* dominant)	**F2 (*Bifidobacterium* dominant)**
Age (y)	29	34
Gender	Female	Male
Height (cm)	156	168
Weight (kg)	61	65
BMI (kg/m^2)	25	23
County	Tanzania	Thailand

5.3.2. Preparations of Thai sticky and jasmine rice powder

Thai sticky rice was obtained from the department of crop science at Kasetsart University in Thailand, while jasmine rice was bought from Asian supermarket in Kitakyushu. The rice was measured 80gm by OHAUS EX224G/AD and soaked into 80mls of water in the cooker for 30 minutes. Then, rice was boiled with the same soaked water until became dry. It was then moved in the top under the rice cooker cover, while boiled water was added in the cooker, steam-cooked till the rice was done. The cooked rice was then transferred into falcon tubes and frozen at -80°C overnight. On the next day, the frozen rice was lyophilized using the FDU-1200 model freeze drier. The freeze-dried

rice was then ground by pestle to obtain fine powder which was kept in small plastic bags and stored in the desiccator for moisture prevention until was used (Fig. 5.2.) [11].

5.3.3. Medium preparation for in vitro culture

In vitro stool culture was performed according to a protocol modified from the method described in the previous paper [13]. Two 100-ml Duran bottles were supplemented with Basal Culture Medium (BCM) added by 1% w/v of sticky and jasmine rice powder. The BCM contained, peptone water at 2 g l^{-1}, yeast extract at 2 g l^{-1}, NaCl at 0.1 g l^{-1}, K_2HPO_4 at 40 mg l^{-1}, KH_2PO_4 at 40 mg l^{-1}, $MgSO_4 \cdot 7H_2O$ at 10 mg l^{-1}, $CaCl_2 \cdot 6H_2O$ at 10 mg l^{-1}, $NaHCO_3$ at 2 g l^{-1}, L-cysteine at 0.05%, bile salts at 0.5 g l^{-1}, vitamin K at 10 μ l l^{-1}, Tween 80 at 2 ml l^{-1} and hemin at 5 mgl^{-1}, and was adjusted to pH 7.4). For the negative control, the third bottle contained only BCM medium with no rice supplement. The medium was autoclaved (121°C for 15 minutes), left to cool to about 50°C. It was moved to the Anaerobic (inoculation) chamber, and the mixtures were transferred into respective fermenter jar connected with pH probe as well as 1M NaOH for pH control.

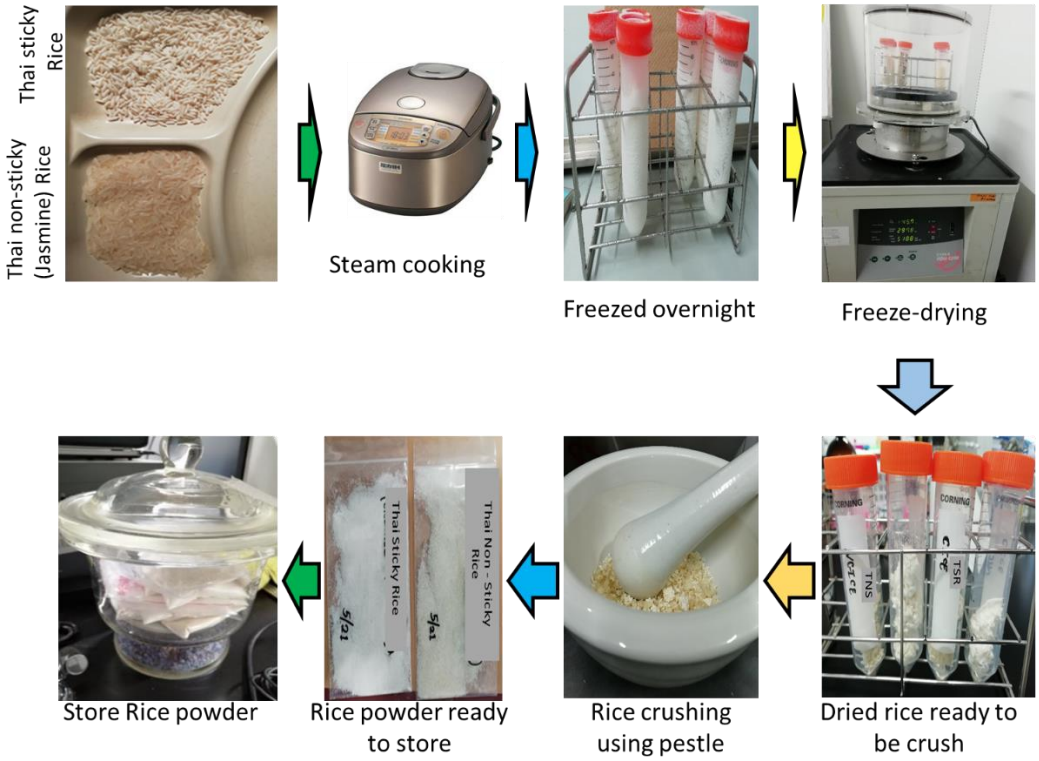

Figure 5.2. Procedures for the preparation of sticky and jasmine rice powder

5.3.4. Single batch fermentation model with feces inoculant and rice powder

The author conducted single batch culture according to the method of reference [14] with some modification including sampling time interval, fecal inoculant diluted with fresh BCM media and inoculant used directly without prior storage. The single batch fermenter was connected by a 200ml bottle of NaOH and pH probe to control pH in the culture medium, also connected by the tube supplying the mixture gas of N_2 and CO_2 (80:20) to maintain anaerobic condition. Fermentation was commenced by inoculating fecal suspension by 5% v/v into each three fermenter vessels of 100 ml working volume. The 5% v/v fecal suspension was prepared by swab collecting feces was

dipped into falcon tube with 15 ml of fresh BCM media, vortexed and then 5ml of fecal suspension was inoculated on each 100ml-fermenter. The gas was purged during fermentation at a rate of 15ml/min and the pH was adjusted automatically to 6.5 by the addition of 1M NaOH. Sample were collected at 0, 4, 8, 12, 24h after the inoculation for 16S rRNA gene sequencing, and SCFA quantification (Figure 5.3) and stored at -80°C before processing.

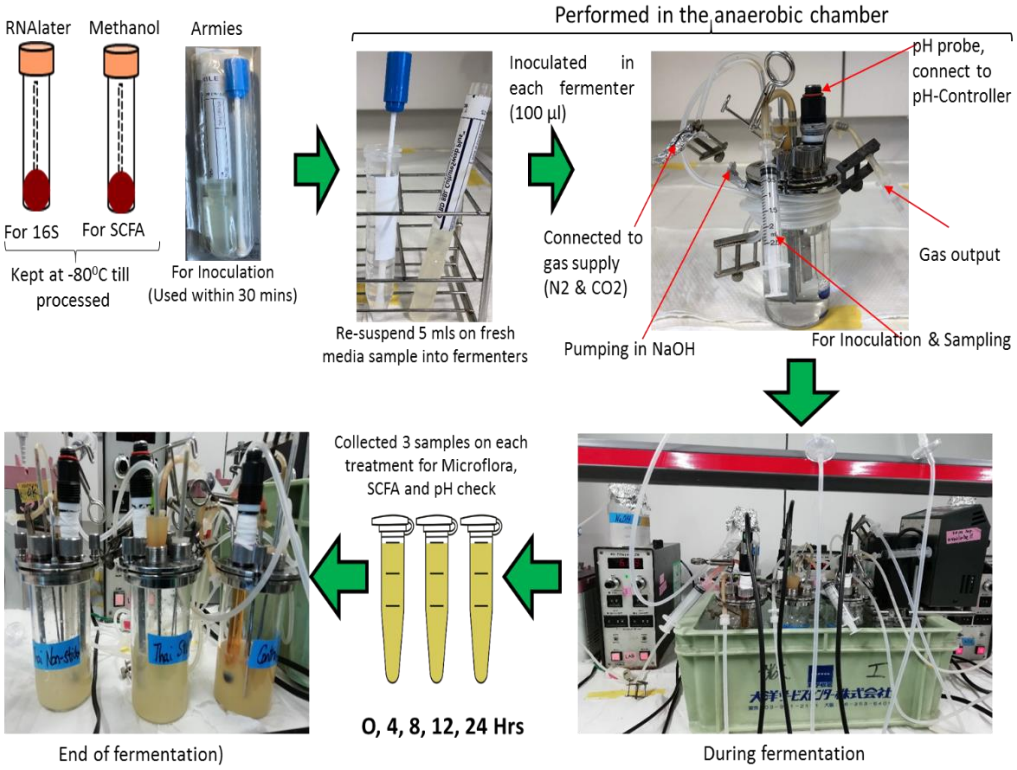

Figure 5.3. Procedure sequence of in-vitro fecal fermentation

5.3.5. pH measurement

Soon after collection, the culture broth was centrifuged at 9000xg for 5 minutes at 4°C and pH of the supernatant was measured by pH metre (DKK-TOA corporation Japan version HM-41X) [13].

5.3.6. DNA extraction and 16S rRNA gene amplicon sequencing

The extraction of bacterial DNA from in vitro culture was performed as in 3.3.3 with few modifications. Briefly, the collected culture sample was thawed and then centrifugation at 9000x*g* for 5 minutes at 4°C, and thereafter**,** 200µl of supernatant was taken and subjected into the phenol-chloroform beads extraction methods to yield total bacterial DNA. Then 16S rRNA gene was amplified and applied to the amplicon sequencing using MiSeq sequencer, as described in 3.3.4 chapter 3.

5.3.7. Processing of 16S rRNA gene amplicon sequences

The paired-end sequences obtained by MiSeq were analysed using the Uparse pipeline in Usearch v9.2 [15]. The OTU sequences were taxonomically assigned by EZbiocloud platform [16]. Furthermore, the taxonomic profile of each sample was determined by QIIME in ver1.8.0.

5.3.8. Quantification of short-chain fatty acid

The collected culture broth was centrifuged at 9000xg for 10 minutes at 4°C and 200 µl of supernatant was transferred into a new Eppendorf tube. Then, 500µl of methanol and 500 µl of chloroform were added to the culture supernatant and vortexed, and centrifuged at 4,600 g for 30 minutes, at 4°C. 300 µl of the aqueous layer was transferred into Amicon ® Ultra Centrifugal filter Ultracel® (Tullagreen, Carrigtwohill, Ireland) and centrifuged at 9,100xg for 3 h, at 4°C. The 300ul filtrate was briefly vortexed for 3 seconds, transferred into the new Eppendorf tube, and applied to integrate Speedvac

concentrator (EYELA FDU-1200, Tokyo Rikakikai Co., LTD., Japan) without heating to evaporate the solvent. 700 µl of NMR solution (PBS/D20 buffer 0.1M, pH 7.4 containing 0.4 M of TSP [Sodium-3-(Trimethylsilyl) propionate-2,2,3,3,d4] was added to the dried pellets to resolve the extracted metabolites. The sample was then centrifuged 9,100xg at 4°C for 10 minutes. Finally, the solution was filled into NMR tube (Hilgenberg GmbH, German) and stored at 4°C until NMR measurement.

5.3.9. Quantification of SCFAs by ^{1}H NMR

The 400MHz ^{1}H-NMR was quantitatively measured at 25°C on a JNM-ECZ400S (JEOL Ltd., Tokyo, Japan). The spectrum was obtained by a standard ^{1}H-NMR pulse sequence with 90° pulse and 10 s delay time while suppressing water signal by using a presaturation method. The number of scans was 64.

The obtained FID signal was subject to Fourier Transformation to yield the ^{1}H NMR spectra, thereafter manually phased, baseline corrected, and integrated in JEOL Delta v5.3.1. The chemical shift and integration were referenced to TSP at 0.00 ppm and 9 protons, respectively. The concentrations of major SCFAs namely acetic acid, proprionic acid, butyric acid and lactate were determined according to the integrations of peaks at the corresponding the chemical shifts (see equation 5.1)

Concentration of metabolite (NMR) =

$$\frac{\text{Sample integrated value X (Concentration of TSP) X (Proton number of TSP)}\ldots}{\text{(TSP integrated value) X (Proton number of peak)}} \qquad \text{Equation 5.1}$$

5.4. Results

5.4.1. Microbial profile fecal inoculant donors

The fecal samples were chosen as inoculant due to their distinct microbiome profile (figure 5.4.). F1 was characterized by abundances of *Bacteroidetes* phylum with *prevotellaceae* family dominance while microbiota of F2 was dominated by *bifidobacteriaceae* family of *Actinobacteria* phylum.

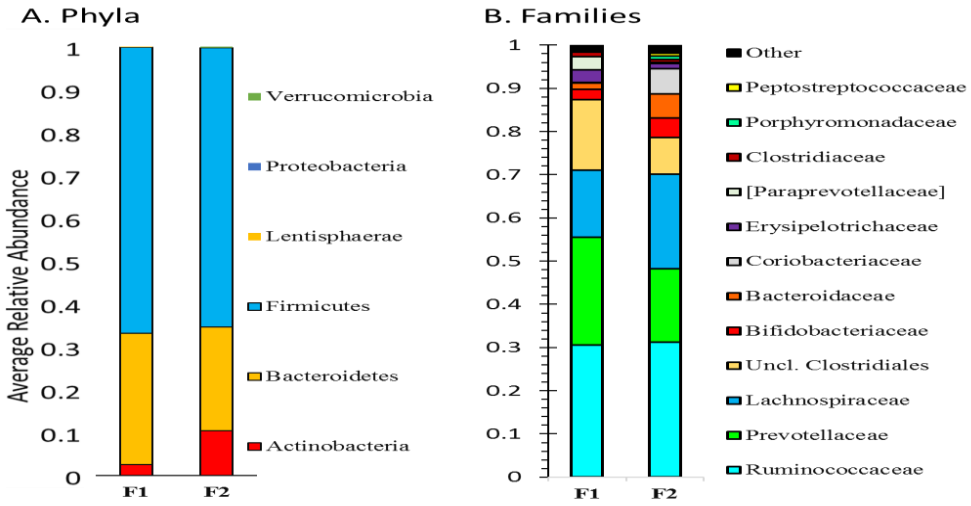

Figure 5.4. Microbial composition of the fecal inoculant (F1 and F2)

5.4.2. Characteristic of bacterial composition of fecal inoculant

The alpha diversity of these two fecal inoculants was calculated based on their OTU composition. As a result, the alpha diversity indices namely, Chao1 (species richness), Shannon (species abundance) and (Observed species), were significantly higher in the F2 donor than the F1 donor (Figure 5.5.A)

Taxonomic compositions of the two inoculants were analysed. The OTUs accounting for more than 1% of total community were shown in Figure 5.5.B. *Firmicutes*

prevailed in both inoculants, while trade-off of some taxonomic groups was observed between these two inoculants, such as genera *Collinsella* and *Eubacterium_g24* dominated in F2 and *Alloprevotella* and *Coprococcus eutactus* dominated in F1 inoculant.

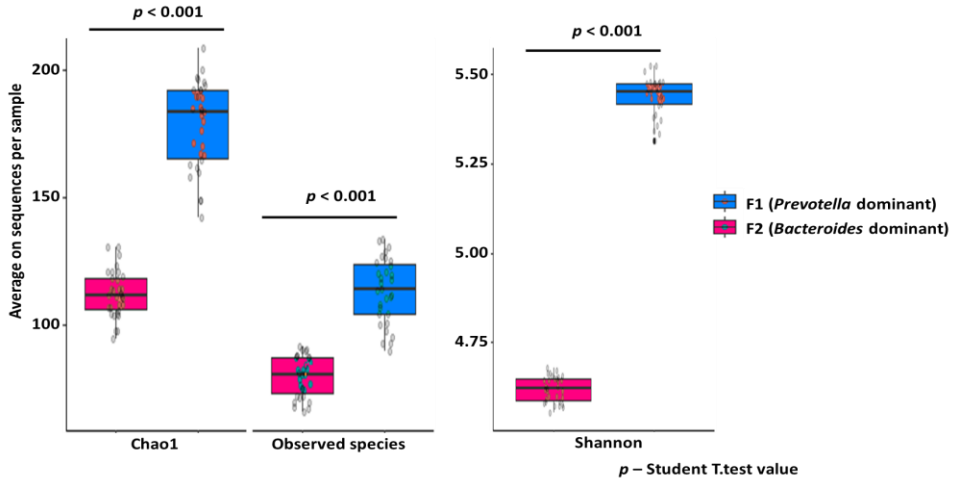

Figure 5.5. A. Alpha diversity metrics of two inoculants. Alpha diversity was calculated from the triplicate sample in 1000 iterations (P-value was calculated by Welch's T.Test).

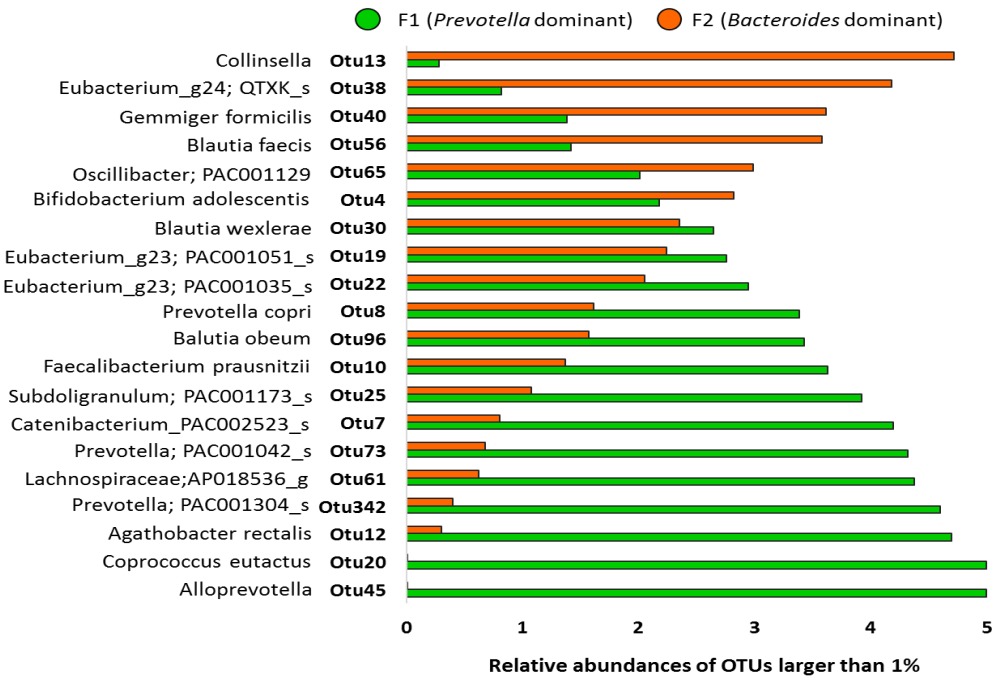

Figure 5.5. B. Relative abundances of dominant OTUs in each inoculant. The OTUs accounting for higher than 1% of total community were shown (P-value was calculated by Welch's T.Test).

5.4.3. pH change profile in the in vitro fermentation

The in vitro culture was performed with combination of rice and inoculant. Either of two types of rice, namely sticky rice powder and jasmine rice powder, was added to the medium and was inoculated by one of the two stool samples; for the control, no rice powder was added. During the fermentation, the pH was kept mostly constant but was slightly decreasing over time (7.4 – 6.5) in some fermentation, suggesting the validity of the pH control system (figure 5.6).

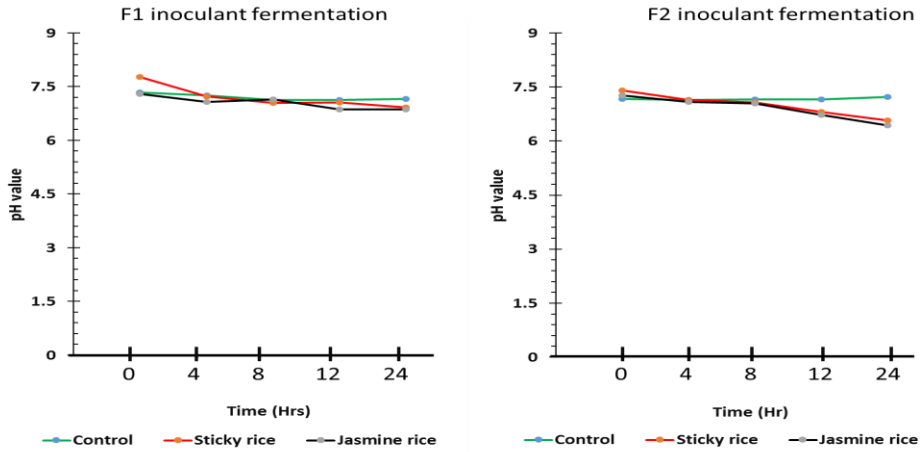

Figure 5.6. Succession of pH during fermentation of both F1 and F2 inoculants. The fermentation was performed in triplicate for each inoculant.

5.4.4. Change in bacteria composition in the in vitro rice fermentation

5.4.4.1. Change in alpha diversity

The successions of alpha diversities, namely chao1 (species richness) and observed_species, were plotted for each fermentation. When inoculated by F2 as well as negative control, alpha diversities tended to decrease for the first eight hours, while the decrease of alpha diversity appeared from 8 to 12 h after inoculation of F1 feces (figure 5.7. A. to D.). All the in vitro rice fermentation by fecal inoculant was conducted in triplicate on both F1 and F2 inoculant in both control, jasmine rice, and sticky rice.

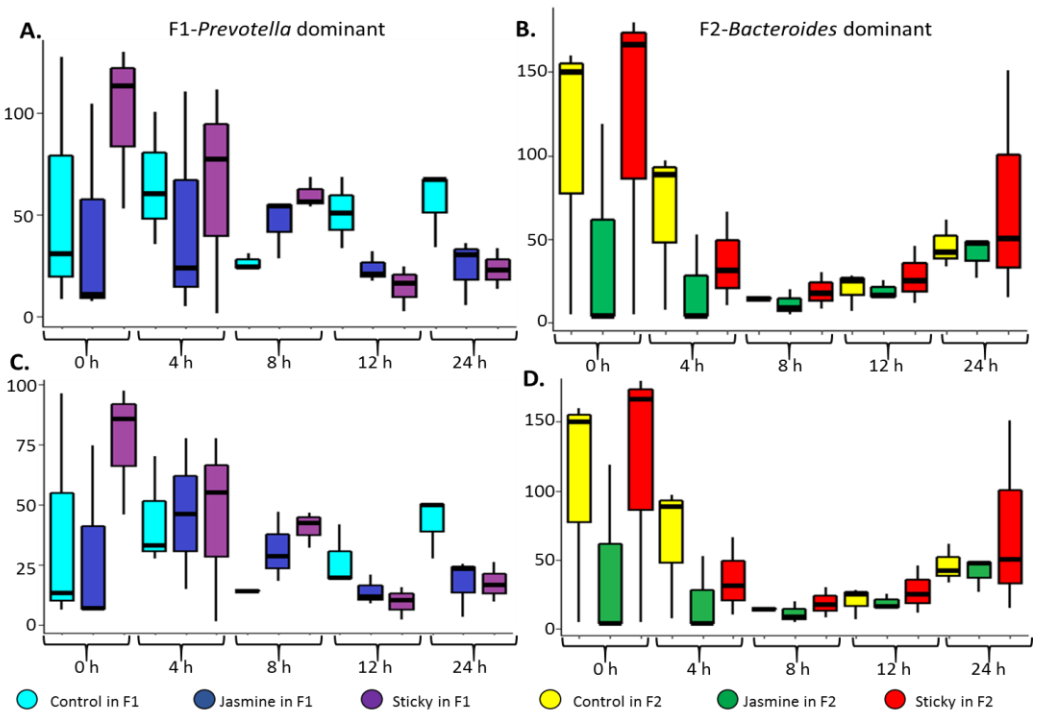

Figure 5.7. A.-D. Succession of alpha diversity indices (Chao1 and observed_species) during rice fermentation. Two different types of rice, namely sticky rice and jasmine rice

were added to the medium. For the negative control, no rice was added. The media were inoculated by the two fecal inoculants and anaerobically incubated for fermentation.

5.4.4.2. Microbial community shift during fermentation of sticky and jasmine rice

The succession of bacteria during composition during fermentation was graphed at the phylum and family level in (figure 5.8. A. and B.). All fecal inoculants were dominated by the major phyla, namely *Firmicutes*, *Bacteroidetes*, and *Actinobacteria*, while F2 inoculant had more Actinobacteria than F1 inoculant. At family level, F1 inoculant contained more *Prevotellaceae* and unclassified *Clostridiaceae* and F2 inoculant contained more *Bifidobacterium*, *Bacteroides*, and *Coriobacteriaceae* compare with other (figure 5.8.A. and B.).

Immediately after inoculation, there was a low level of these major bacteria phyla which might be due to low concentration of fecal inoculant used, but at 24 hours, there was a robust shift of *Actinobacteria* and *Bacteroidetes* observed in both jasmine and sticky rice under F1 inoculant fermentation. Despite the same basal medium used, but the changes in *Actinobacteria* and *Bacteroidetes* was low in F2 inoculant, although not significant (figure 5.8. A). The level of the same phyla in the F2 inoculant was small, although not significantly different.

Interestingly, we observed some bacterial community trend of relations during fermentation at family level involved F1 inoculant on both rice treatments. In jasmine rice, bacteria belonging to family *Peptostreptococcaceae* steadily decreased while *Clostridiaceae* increased in abundance until the last hour of fermentation. Whereas,

sticky rice demonstrated a clear difference in which family *Peptostreptococcaceae* increased with the increase in *Clostridiaceae* until 12 hours, but, both families decreased at 24 hours as *Actinobacteria* family powerful dominated in abundance (Fig. 5.. B). The F2 inoculant shows a trend can be noticed but not consistent and is somewhat different from the F1 inoculant (Fig. 5.8. B).

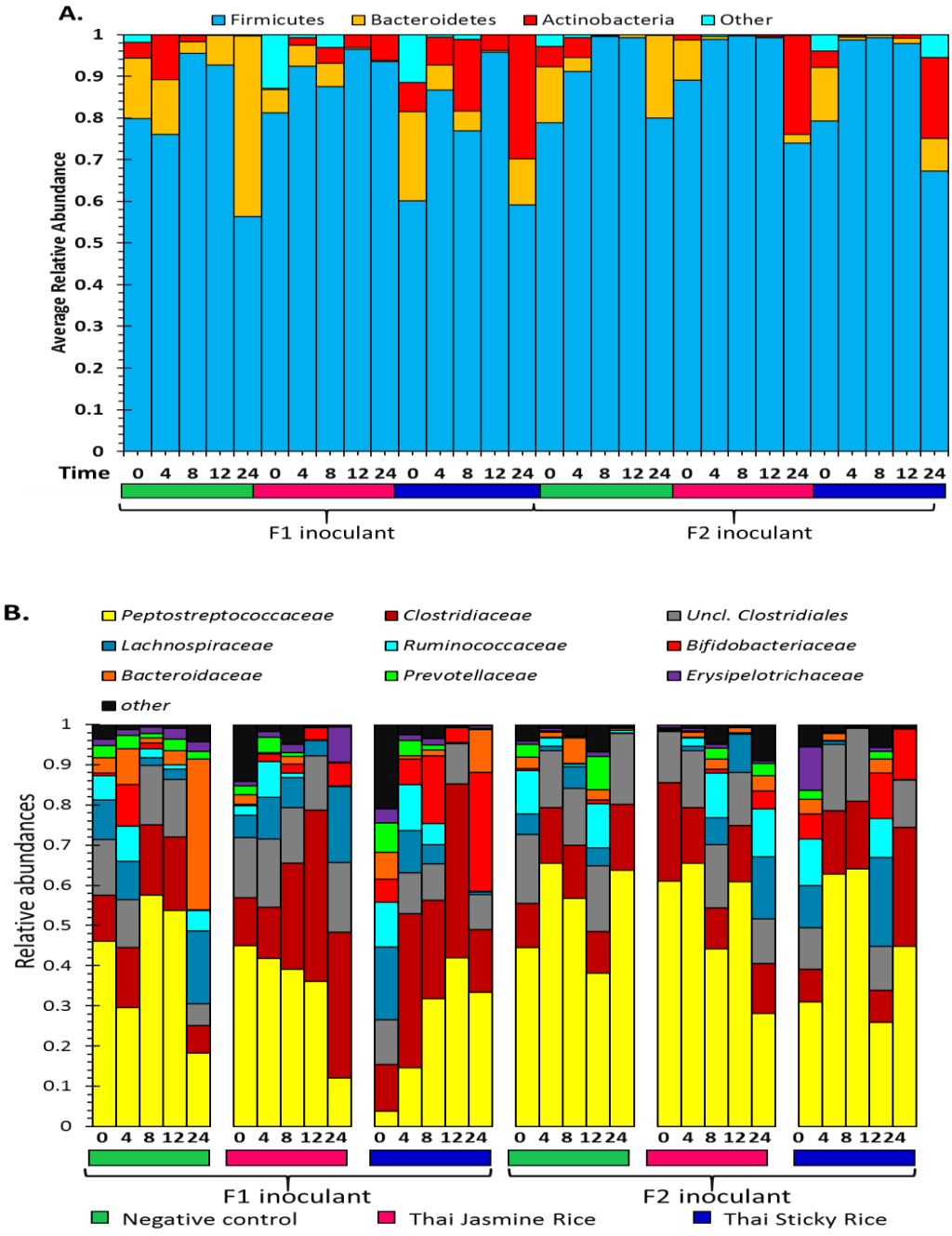

Figure 5.8. Relative abundances of bacteria at (**A**) phylum and (**B**) family's level. Average of three runs on each fermentation were graphed.

5.4.4.4. Changes in the ration of Bacteroidetes, Actinobacteria and Firmicutes

In all batches the ratio of *Bacteroidetes* to *Firmicutes* was decreasing until 12 h and then increased to 24 h (figure 5.9. A. and B.). The increase of *Bacteroidetes* in later phase was more in control batch than in rice batches, suggesting that *Bacteroidetes* grow more competitive in non-carbohydrate condition. The later-phase increase of *Bacteroidetes* was much less in Jasmine rice batch, while it was evident in sticky rice batch. This indicates that *Bacteroidetes* favour sticky rice. These tendencies were common in both P- and B-inoculant batches (figure 5.9. A. and B.).

On the other hand, in the control batches ratio of *Actinobacteria* to *Firmicutes* decreased to the end of fermentation for both inoculants. The ratio of *Actinobacteria* increased sharply at 24 h in F2 inoculant for both jasmine and sticky rice fermentation while F1 inoculant favoured only sticky rice (figure 5.9. C. and D.).

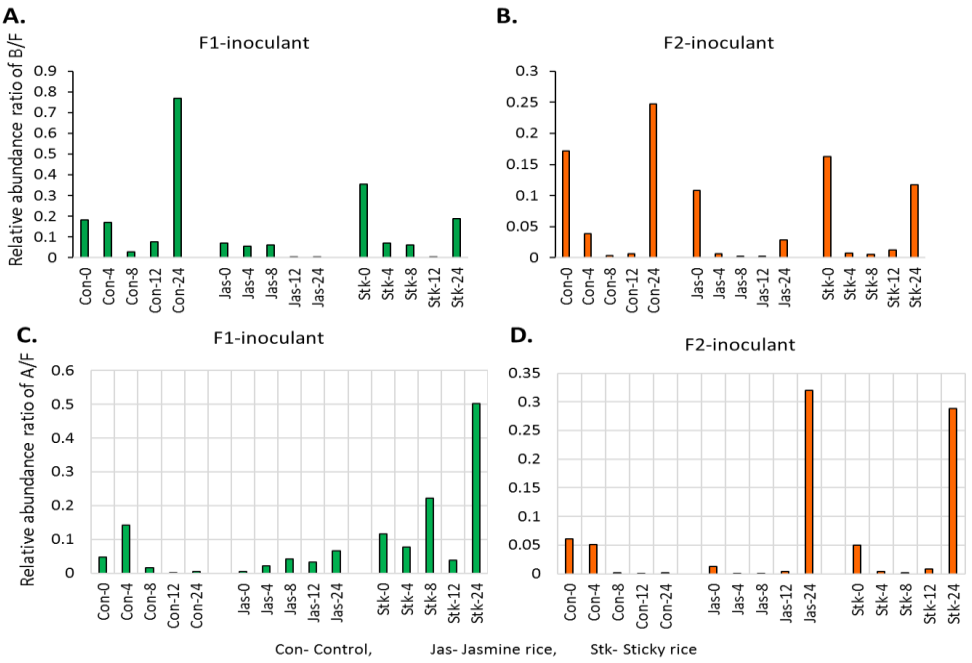

Figure 5.9. Change in ratio of *Bacteroidetes* to *Firmicutes* (A. and B.) *Actinobacteria* to *Firmicutes* (C. and D.) during in vitro fermentation.

5.4.4.5. SCFAs production in in vitro fermentation

SCFA concentration were monitored in each fermenter using ^{1}H NMR. Acetate increased gradually after the inoculation, while the other SCFAs started to increase after 12 h. Productions of propionate and butyrate were observed slightly at 24 h, whereas lactate increased drastically after 12 h (figure 5.11), notably in Sticky-rice batch with B-type inoculant which reaches the same level as acetate (figure 5.10). The increase of lactate significantly correlated with the increase of *Bifidobacterium* (p=0.035, rho=0.25) (figure 5.12).

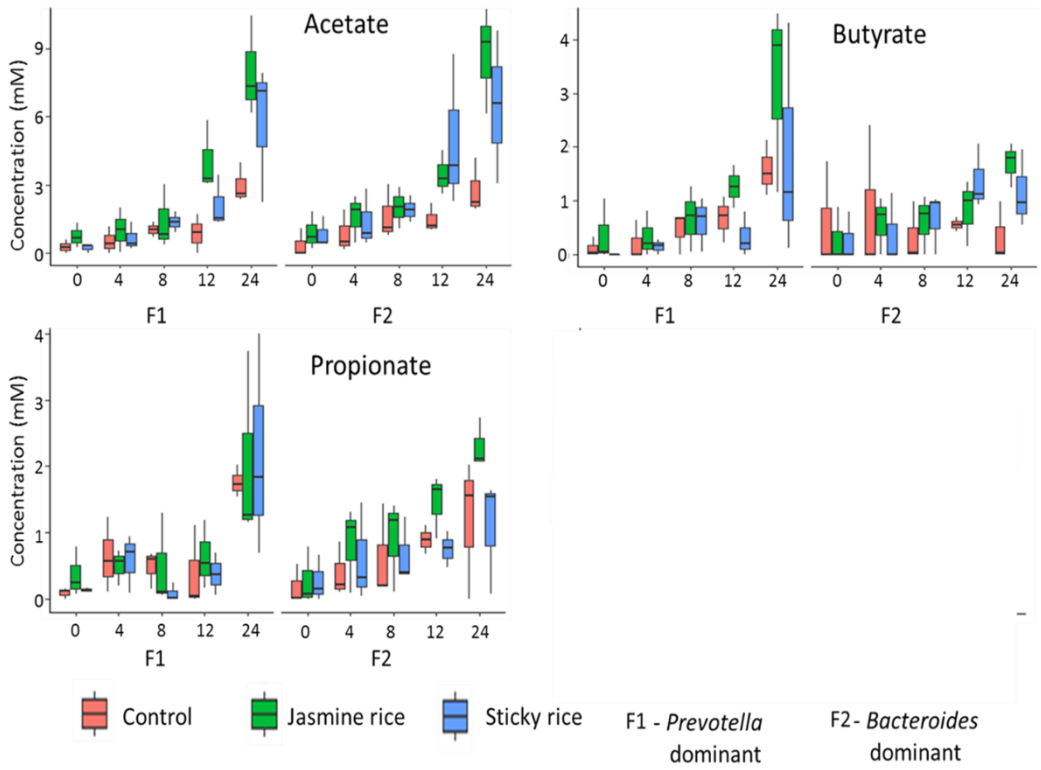

Figure 5.10. Individual SCFA from F1 (P-type) and F2 (B-type) inoculant fermentation

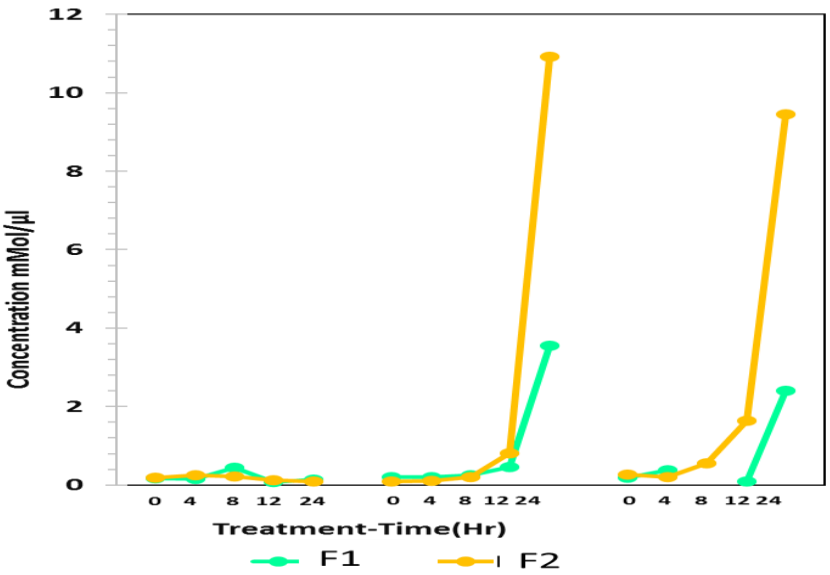

Figure 5.11. Lactate from F1 (P-type) and F2 (B-type) inoculant fermentation

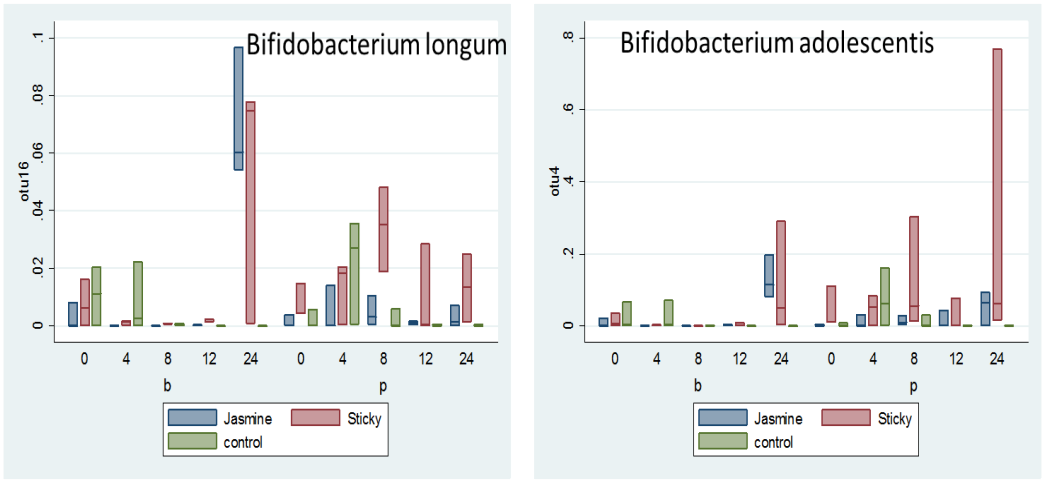

Figure 5.12. *Bifidobacterium* species increased during fermentation with F1 and F2

5.5. Discussion

Rice is the staple food in Thailand, in which a variety of sticky and jasmine rice [10] are consumed in each province [12]. These varieties differ mostly in the main contents of starch, which is made from amylose and amylopectin [17] and dietary fibers, which influences on gut microbiota [18]. Notably, resistant starch must have profound impact on the composition and functionality of gut microbiota [7],[19]. This technique enhances the amount of resistance starch, specifically R3, once consumed by the host and provide the most crucial health benefits [6].

To understand the effect of two different cultivars of rice commonly consumed by Thai people, namely sticky and jasmine rice on the gut of Thai people, the author performed an in vitro fermentation using both sticky and jasmine rice as carbohydrate sources and using two different type of human feces, namely F1 and F2, each including high abundance of *Prevotella* and *Bacteroides* respectively.

The primary findings indicate that both rice powders prepared from jasmine and sticky rice promote the growth of *Bifidobacterium* and its lactate fermentation. Jasmine rice is somewhat favourable for the lactate production but there was no difference found in the growth of *Bifidobacterium*. In this study, the bacteria population was obtained by the 16S rRNA amplicon sequencing that represents only relative abundance not absolute abundance. Quantitative data of bacteria population may link more precisely bacteria growth to SCFA fermentation. It was also found that control batch without extra carbohydrate source promoted the growth of Bacteroides, while sticky rice but not jasmine rice did it even weakly. Phanphen Phoonlabdacha has reported that pregnant mother who daily consumed sticky rice in the north and north-east region in Thailand have a remarkably high abundance of *Bacteroides* in their gut (Ph.D thesis, P.

Phoonlabdacha). Since the *Bacteroides* is taxonomically and phenotypically wide group, notably in terms of carbohydrate digestion activity, we should address species or enzymatic level of *Bacteroides* involved in the sticky rice-dependent growth.

A number of dietary intervention studies, including short and long term studies have their effects on the microbial composition, notably shift of *Bacteroidetes* to Firmicutes ratio [20]–[22]. The fact that shift of the B/F ratio was also observed in the in our in-vitro fermentation with sticky rice may suggest some health benefit of stick rice, although there are a profound number of controversial for the obesity-related B/F-ratio shift and also high glycemic index of sticky rice should be careful considered when consuming rice.

In general, the promotion of *Bifidobacterium* growth is expected to benefit the host health while the health benefit by the *Bacteroides* increases warranty for further study. On top of that, rice consumption for carbohydrates, should be good health cultivar and well prepared to minimize risks associated with Diabetes mellitus [23], [24].

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Chapter 6

Summary and concluding remarks

It is known that a traditional Thai diet is relatively high in vegetables and low in fat. Moreover, their recipes improve health since they lower the disease causative agents like in cancer [7]. However, food modernization is not always benefit in terms of the increase in refined carbohydrates, sugars, fats, animal proteins and synthetic food additives. Previous studies have shown the effect of Western diets (WD), in which, regardless of age, bacteria richness, and diversity were low [2]–[4]. Moreover, WD mainly enriched *Bifidobacterium*, *Bacteroides*, and some firmicutes; in contrarily, traditional diets reserve mostly *Prevotella* [2]–[6].

In this doctoral thesis, the author described the effect of dietary life change, notably loss the Thai traditional diets on Thai gut microbiome.

Chapter 1 provide general idea on the status of Thai gut microbiome and their health and dietary lifestyle.

Chapter 2 indicated the current status of school-age children in urban and rural regions. The Buriram children consumed a traditional diet rich in vegetables and less sugars while Bangkok children consumed diet high in fat, proteins, and less vegetables which reflects the modernization in the city.

Chapter 3 clearly showed the shift in microbial composition in relation to the consumption of different dietary nutrients between urban and rural. The author documented that Bangkok children had high abundance of bacteria commonly higher in Italy, which appears to correspond to gut microbiome of urban children in Thailand. In contrast, the Buriram children harboured more diversified bacteria including beneficial bacteria compared to Bangkok children. The author suggests that the urbanization has a profound influence on gut health of Thai through decrease of their traditional diets.

In chapter 4, the author indicated that Buriram children had a high concentration of both butyrate and propionate in their gut as compared to Bangkok children. These differences in the SCFAs' concentrations reflected to the differences in the bacterial composition between these two cities. The fecal metabolites profiles from two cities were clustered into five metabolotypes each characterized by high abundance of SCFAs and MCFAs (MT1), amino acids (MT2), arginine (MT3), overall, less (MT4), and amino acids and amines (MT5). MT1 mostly comprised of Buriram children, while MT2 mostly comprised of Bangkok children. Further predicted functional metagenomics showed under-representation of eight genes involved in butyrate biosynthesis pathways in MT2. Altogether, it is suggested that the diets low in vegetable and high in fat decrease in colonic short-chain fatty acid fermentation through the alteration of gut microbiome.

In chapter 5, the author monitored the effect of sticky and jasmine rice on the composition of bacteria and short-chain fatty acids for up to 24h in the in vitro culture system. In both media added jasmine and sticky rice powder, lactate sharply increased after 12h with an increase in *Bifidobacterium*, whereas it was not observed in the control batch suggesting *Bifidobacterium* growth with lactate fermentation using rice carbohydrate while the *Bacteroides* favoured sticky rice. The promotion of *Bifidobacterium* is expected to benefit host health, while the health benefit of *Bacteroides* requires further studies.

In conclusion, the decrease of traditional diet in Thailand reduces the benefit of gut microbiome through the alteration of structure and functionality of bacterial community. Furthermore, the author demonstrated the efficacy of in vitro culture model to investigate the effect of food component on the structure and functionality of gut bacterial community. Since it is indispensable to evaluate the functionality of each food

in respect to the benefit to gut microbiota in addition to direct host benefit, it is important to continue in vivo and in vitro studies to investigate the link between diet and gut microbiome.

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List of publications

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