THE EFFECT OF DIET AND BMI ON GUT MICROBIOTA PROFILE AMONG PRIMARY SCHOOL CHILDREN IN KOTA BHARU, KELANTAN

BY

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ABSTRAK

Latar Belakang

Organisma usus manusia wujud dalam pelbagai kepekatan pada permukaan mukosa usus dan memainkan peranan penting dalam menjaga kesihatan. Kegemukan dan pengambilan pemakanan diketahui mempunyai implikasi dalam memacu faktor struktur organisma usus.

Kaedah

Kajian ini meneliti komposisi komuniti organisma usus dalam kalangan pelajar sekolah di Kota Bharu yang berumur dalam lingkungan 7-11 tahun (n=81). Sampel najis diambil dan dibuat analisis jujukan gen 16S rRNA.Tinjauan pemakanan didapatkan bagi menilai perkaitan di antara pemakanan dan organisma usus.

Keputusan

Analisis komposisi bakteria menurut tahap taksonomi (genus,keluarga dan filum) menunjukkan jumlah besar bakteria pada tahap genus adalah *Bacteroides* 23% dan *Prevotella* 22%. Organisma usus tersebut diklasifikasikan kepada dua enterotype seperti kluster, yang mana setiap daripadanya dipacu oleh *Bacteroides* (Jenis B) atau *Prevotella* (Jenis P). Analisis statistik menunjukkan perkaitan signifikan dengan BMI (nilai p=0.005). Kami juga mendapati organisma usus jenis B mempunyai perkaitan positif dengan ayam dan ikan (nilai p=0.007 dan 0.038), dan jenis P mempunyai perkaitan positif dengan buah-buahan, produk tenusu, makanan laut, serbuk perasa dan minuman. (nilai p= 0.025,0.020,0.032, 0.001, dan 0.012)

Kesimpulan

Wujudnya perkaitan di antara BMI dan pemakanan ke atas organisma usus dalam kalangan pelajar sekolah rendah yang sihat dalam populasi Kota Baharu. Kajian seterusnya adalah perlu untuk mengkaji mekanisma di sebalik perubahan ini dan seterusnya kaitannya dengan kesihatan dan penyakit.

ABSTRACT

Background

Human gut microbes are present in large concentration on intestinal mucosal surfaces and play important roles in host health. Obesity and dietary intake are known to have implications in driving factors for structure of gut microbiota.

Method

The present study examined the composition of the gut microbial community among primary school children in Kota Bharu, aged 7-11 years old (n=81). Fecal sample were collected and subjected to 16S rRNA gene sequencing analysis. Dietary survey were obtained in order to assessed the association of diet between gut microbiota.

Result

Analysis of bacterial composition according to taxonomic rank (genus, family and phylum) revealed most abundance of bacterial at genus level were *Bacteroides* 23% and *Prevotella* 22 % respectively. The microbiota were classified into two enterotype like clusters , each driven by *Bacteroides* (B-Type) or *Prevotella* (P-Type). Statistical analysis revealed B-Type and P-Type shows significant association with BMI (*p* value =0.005).We also found that B-Type of microbiota positively associated with chicken and fish (*p* value = 0.007 and 0.038 respectively), whereas P-Type showed positively associated with fruit, milk & dairy product, seafood, seasoning & flavourings and beverage (*p* value = 0.025, 0.020, 0.032, 0.001 and 0.012 respectively).

Conclusion

There was an association between BMI and diet on gut microbiota among healthy primary school children in the Kota Bharu population. Further studies are necessary to elucidate the mechanism behind these changes and ultimately their link to health and disease.

CHAPTER 1

LITERATURE REVIEW

Human gut microbiota is present in large concentration in the human intestinal mucosa surface and play important roles in managing the health and diseases of the host as it closely associated with the host mucosal surface and interact with the host (1) . Maintenance of the gut microbiota balance creates a beneficial symbiotic relationship responsible for dietary energy extraction, as well as a multitude of other processes (2).

The main contributing factor for composition of human gut microbiota is dietary habit (3). An alteration of gut microbiota due to dietary change has the potential to profoundly affect host's health and development.(4,5,). Dietary habit can structure the gut microbial community by supplying the nutrients and conditioning the intestinal microenvironment (1,3,6,7). The contributor for growing epidemic of chronic illness in the developed world, including obesity and inflamatory bowel disease were induced by changes of diet to gut associated microbial communities. (8).

The gut microbiota also had involved in the control of body weight and energy homeostasis. Studies have shown that microbial community in the human intestine may play an important role in pathogenesis of obesity (9) .Information regarding composition and function of the gut microbiota during childhood is limited. Some evidence showed gut microbiota reaches a relatively stable adult like state in the 3 years of life while other studies showed that it continues to develop through adolescence. (10,11).

The 16S rRNA gene sequencing techniques have been used to detect human gut microbiota. Previous studies show the existence of at least two types of microbiomes comprising a trade-off of Prevotella or Bacteroides within or across cohort, or further within

individual over time (6,7,12-15). Bacteroides- dominated enterotype high in animal fat diet, whereas a carbohydrate-rich diet is associated with the prevotella- dominated enterotype.

Obesity is a multifactorial disease that predisposes various diseases (23), and according to World Health Organisation, obesity is considered as global epidemic disease(19). Excessive food intake, especially of high-fat and sugar products, together with insufficient excercise and genetic susceptibility, are considered risk factors for developing obesity. An alteration in gut microbiota have been associated with the development of obesity (21,22). A high fat diet may contribute to imbalances in the gut microbiota and disrupt the gut barrier integrity, leading to increased endotoxaemia and metabolic diseases (23). In addition, the efficiency of food conversion in obese individuals is higher and thus provides the host with a greater amount of usable energy in the form of short chain fatty acids (SCFAs) , which contribute to adiposity, insulin resistance and type 2 diabetes (24) .

Childhood life may provide opportunities for microbiome intervention to promote health or to prevent disease (26). Therefore, it is vital to establish a baseline understanding of gut microbiome structure and function among paediatric population, in which it varies and is unique among healthy children as opposed to infancy, when digestive function is immature or throughout adulthood , presumed to be matured.(27,28) .

CHAPTER 2

OBJECTIVE OF THE STUDY

2.1 General Objective

1) To determine the basal Gut Microbiota profile among healthy primary school children of 7-11 years in Kota Bharu, Kelantan.

2.2 Specific Objectives

1) To determine association of demographic data (age, gender and race) between *Bacteroides* and *Prevotella* among healthy primary school children
2) To determine association of BMI between *Bacteroides* and *Prevotella* among healthy primary school children after controlling the effect of subject demographic and diet.
3) To determine association of dietary intake between *Bacteroides* and *Prevotella* among healthy primary school children after controlling the effect of subject demographic and BMI.

CHAPTER 3

MANUSCRIPT

3.1 **The effect of diet and BMI on gut microbiota profile among primary school children in Kota Bharu, Kelantan.**

Running title: The effect of diet and BMI on basal gut microbiota

Journal: Environmental Microbiology

3.2 **ABSTRACT**

Human gut microbes are present in large concentration of intestinal mucosal surface and play important roles in host health. Obesity and dietary intake known to have implication in driving factor for structure of gut microbiota. The present study examined the composition of the gut microbial community among primary school children in Kota Baharu, aged 7-11 years old (n=81). Fecal sample were collected and subjected 16S rRNA gene sequencing analysis. Dietary survey were obtained in order to assessed the association of diet and gut microbiota. Analysis of bacterial composition according to taxonomic rank (genus, family and phylum) revealed most abundance of bacterial at genus level were *Bacteroides* 23% and *Prevotella* 22 % respectively. The microbiota were classified into two enterotype like clusters , each driven by *Bacteroides* (B-Type) or *Prevotella* (P-Type), respectively. Statistical analysis revealed B-Type and P-Type shows significant association with BMI (*p* value =0.005). We also found that B-Type of microbiota is positively associated with chicken and fish (*p* value = 0.007 and 0.038 respectively), whereas P-Type showed positive association with fruit, milk & dairy product, seafood, seasoning & flavourings and beverage (*p* value = 0.025, 0.020, 0.032, 0.001 and 0.012 respectively).

There was a presence of an association between BMI and diet on gut microbiota among healthy primary school children in Kota Baharu population. Further studies are necessary to elucidate the mechanism behind these changes and ultimately their link to health and disease.

Key words: diet, BMI, gut microbiota

3.3 **INTRODUCTION**

The gut microbiota are the microorganisms that inhabit the human gut detected using novel sequencing technology. The desired outcome of the gut colonization process is a complex microbial community that provides the barrier against foreign microbes and some harmful components in the diet. Additionally, the colonization process creates the basis for the establishment of a 'noninflammatory' status of the gut. The collective composition of the colonizing strains maintains a healthy gut microbiota, as the development of the disease-free state of the gut lies in the host microbe interaction. There are many documented evidences which demonstrated that disturbance of intestinal microbiota is linked to the risk of developing infectious, inflammatory and allergic diseases. It is of great interest to characterize both composition and succession of the intestinal microbiota.

The 16S rRNA gene sequencing technology is considered the gold standard for phylogenetic studies of microbial communities and for assigning taxonomic names to bacteria (29). Several studies of the human gut microbiome that using this technique, reported species diversity between individual. Although it varies between individual, the concept of "enterotypes" has been proposed, in which the gut microbial community structures of adult human beeings are classified into three types, each defined by high abundance of Bacteroides, Prevotella, and Ruminococcus (30).

There are multiple factors that influence the structure of GI tract microbiota, which include microbes acquired at birth, diet, host genetic & physiology, drug intake and disease (15). Dietary intake is the one of the major contributor factor for gut microbiota community as it provides nutrition and alters the environment for the microbes (3). In particular, alterations in the intake of carbohydrates, proteins and fats can significantly affect the composition of the microbiota (34). Fermentation of dietary fibre and slowly digestible carbohydrates by gut microbiota forms a range of bacterial metabolites, including short chain fatty acids (SCFAs), typically acetate,propionate and butyrate, which these metabolites represent of additional energy source for the host (1).

Such perspective studies provide markers for the stage of health and positive guidance for microbial colonization through dietary interference. Therefore, the objectives of our study were to characterize the gut microbiota profile among healthy youngster in our population and to determine the association between BMI and dietary intake in relations to gut microbiota . We performed an analysis of the gut microbiota in 81 subjects among primary school childrens in Kota Bharu, Kelantan, aged 7-11 years old. Sequencing the 16S rRNA genes obtained from fecal samples was performed to obtain an overall picture of the gut bacterial composition of the subjects according to taxonomic rank. Finally we performed statistical analyses to determine the association between BMI and dietary intake with gut microbiota. From this study, we can provide the background for further perspective studies of disease population and age groups.

3.4 **METHODOLOGY**

Study design and population

The present study was a cross- sectional study involving primary school students in Kota Baharu aged between 7-11 years old. Based on the state education department database, there are 95 primary schools in Kota Bharu Kelantan, Six primary schools were selected using simple random sampling in which 3 Malay schools and 3 Chinese schools were selected. From each school, the subjects were selected through systematic sampling. A total of 81 students were enrolled in this study, which comprised of 44 male and 37 female students. Thirty-six of them were from Malay schools and 45 other students were from Chinese schools. They had not contracted any infectious disease that required medical attention 3 months prior to the sampling process. For those who were taking antibiotic within 3 months and had been taking probiotic/ prebiotic product diets from 2 weeks before sampling were excluded in this study. Children aged 7 to 11 years old were chosen due to the following two reason: i) gut microbiota of this age was reported to be associated with adult-like configuration deviating from infant micriobiota (35,36,37) and ii) children of this age mainly consumed food prepared at home, with their diet consisting of traditional foods, and their dietary profiles are more uniformed and can be accurately tracked(15).This study was approved by the Universiti Sains Malaysia (USM) Human Research Ethics Committee (USM/JEPeM/15110494) which complied with acceptable international standards including the Declaration of Helsinki. Written informed consents were obtained from the parents for all enrolled subjects.

Sample collection and processing

The parents/guardians self-administered a validated Malay-langueage questionnaire (food frequency questionnaire, FFQ) that addressed dietary intake in the past 12 months before stool

sampling. The questionnaire consists of a list of food and beverages with response categories to indicate usual frequency of consumption over the period of interest that included 130 foods and drink. Demographic data including age, gender, ethnic group, weight and height were also captured in the questionnaire. The children were classified as underweight, normal weight, overweight or obese, based on gender and age-specified BMI percentiles from the Centers for Disease Control (http://www.cdc.gov), in which obesity is defined as $\geq 95^{th}$ percentile and normal-weight defined as $\geq 5^{th}$ percentile and $< 85^{th}$percentile. The BMI was calculated by weight (Kg)/height2 (m2).

The fecal samples were collected at household level by each participating child with the help of their parents/guardian. Technique for fecal collection is shown in supplementary method. Fresh feces were collected in sterile feces containers and then suspended into 2ml RNAlater® reagent (the reagent for stabilizing the nucleic acid) and were stored at room temperature. The samples were transferred to the laboratory within 12 h, stored at 4uC, and used for extracting DNA within four weeks. Previous studies have shown that the bacterial composition data did not change in four weeks under the storage conditions used (15).

DNA extraction

The total bacterial DNA extraction was performed using QIAamp® Fast DNA stool mini kit (https://www.qiagen.com) with minor modification. Purification of DNA from stool samples was automated on the QIAcube . The detail protocol is described in supplementary methods.

Next- generation sequencing

The concentration of the extracted double-stranded DNA was measured using the Quant-iTTM Picrogreen® kit. After quantification, the concentration of the DNA was normalised to 12.5 ng with autoclaved MilliQ water to be used later for the polymerase chain reaction (PCR) to produce 16S rRNA DNA amplicon. KAPA HiFiTM PCR Kit was used in the PCR

for 16S rRNA DNA amplicon production. The PCR was done in a thermocycler with the following thermocycling conditions: 95°C for 5 minutes for the initial denaturation step, followed by 25 cycles of subsequent denaturation at 95°C for 30 seconds, primer annealing at 55°C for 30 seconds and DNA extension at 72°C for 30 seconds. The final extension cycle was at 72°C for 5 minutes. After which , the DNA was kept at 4°C for later use.

The PCR products were then purified using Agencourt® AMPure® XP beads. The purified DNA amplicons were then suspended with 50 µl of 10 mM Tris buffer (pH 8.5). In the index PCR, the KAPA HiFiTM PCR Kit was used again for the attachment of dual indices and Illumina sequencing adaptor sequences to the amplicons produced in the previous PCR. The reaction mixture for each of the DNA samples included 5 µl of the DNA amplicons produced in the previous PCR, 5 µl of i7 (Index 1) and i5 (Index 2) primers each and 12.5 µl of 2x KAPA HotStart Ready Mix. This was done on a 96-well plate and each well containing one sample library will have a unique i7 and i5 index pair. The PCR was done in the thermocycler with the following thermocycling conditions: initial denaturation at 95°C for 3 minutes, followed by 8 cycles of denaturation at 95°C for 30 minutes, annealing of indices and adaptor sequences at 55°C for 30 seconds and extension at 72°C for 30 seconds. The final extension was at 72°C for 5 minutes.

The DNA libraries produced were purified again using the Agencourt® AMPure® XP beads and were resuspended with 25 µl of 10mM Tris buffer (pH 8.5). Quantification of the library concentration for each sample was again measured with the Quant-iTTM Picrogreen® kit. Each of the sample libraries was then normalised to 4nM using 10 mM Tris buffer (pH 8.5). After which, 5 µl of each library was aliquoted into a single tube with the resulting being the Pooled Amplicon Library. The PAL was then re-quantified with qPCR using the KAPA Library Quantification Kit.

The PAL was mixed thoroughly and a small proportion was taken out to be used for sequencing. 0.2 N sodium hydroxide (NaOH) was added to the PAL to denature the DNA. The solution was lightly vortexed for a short while, centrifuged at low speed and incubated at room temperature for 5 minutes for the denaturation process to occur. The result is a denatured amplicon library. After which, pre-chilled hybridization buffer (HT1) was added to the DAL, resulting in a diluted DAL in 1mM NaOH. The DAL is usually diluted to 6 pM with pre-chilled HT1. As for the preparation of the PhiX control, the denaturation steps were the same. The denatured PhiX library was then diluted to the same concentration as the diluted DAL. Both diluted DAL and PhiX control were kept on ice till they were ready to be combined. DAL is combined with the denatured PhiX control in a tube. The tube was then incubated at 96°C for heat denaturation. After 2 minutes, the tubes were taken out, inverted to mix, then immediately placed in an ice water bath for 5 minutes (Illumina©, 2013).

- DNA next-generation sequencing with Illumina© MiSeq Desktop Sequencer

The solution inside the tube containing the DAL and PhiX control was loaded into the Miseq Reagent v2 run cartridge that was pre-thawed at room temperature. The cartridge was then inserted into the Illumina© Miseq Desktop Sequencer and the flow cell was also loaded for sequencing to start. The 16S rRNA DNA sequence data was obtained after approximately 39 to 45 hours.

Analysis of pyrosequencing data

The sequence data obtained was analysed using QIIME (Quantitative Insights Into Microbial Ecology) version 1.9.1. Using the QIIME script, the forward and reverse reads of the same sample were first joined. The paired reads were then filtered based on their quality, which was determined by their Q-score. Those with a Q-score of 25 were selected. Chimeric sequences have a low Q-score and hence were filtered out and removed using USEARCH v6.1.

Determination of operational taxonomic units (OUT) & taxonomic classification

After using Greengenes v13.8 reference database, open-reference operational taxonomic units (OTUs) were picked out from the resultant. In this step, sequences that had a 97% similarity to the sequences in the Greengenes v13.8 database were clustered into different OTUs. The OTUs were summarized into taxa and taxonomy plots so that the profile of the bacteria in each sample can be elucidated. Rarefied OTU tables were generated and alpha diversity metrics were computed for each rarefied OTU table.

Beta diversity between the samples were calculated using the UniFrac distance matrices, generating the three-dimensional Principal Coordinate Analysis plots that were visualised using Emperor. UniFrac produces distance matrices between the samples and was used to derive the beta diversity of the samples. Both unweighted and weighted UniFrac were used. Weighted UniFrac uses quantitative measures, which is more ideal in revealing community differences that are due to relative taxon abundance changes. On the other hand, unweighted UniFrac uses qualitative measures, which is informative on the differences in the taxa present in the community.

Statistical analysis

Statistical analysis was performed with IBM SPSS version 22 software. Sociodemographic and physical characteristic of participants (age, gender, races, weight, height, and BMI) were tabulated for descriptive statistics. All categorical variables were described in frequency and percentage. Numerical data were described in mean and standard deviation (SD). Binary logistic regression analysis (odd ratio[OR] and 95% confidence interval [CI] was used to test for factors associated with *Bacteroides* and *Prevotella* type of microbiota among healthy primary school children. A P value < 0.05 was considered as significant.

3.5 RESULT

Subject characteristic

Of 160 subjects were screened, only 81 subjects had complete data and were included in data analysis, the remaining 79 subjects were absent during measurement day for various reasons. **Table 1** shows the socio-demographic and physical characteristics of the participants. This study comprises of 81 students which majority were in standard 4 with 24.7% followed by standard 2 (22.2%) and standard 5 with 19.8%. More than half of total students were Chinese student with 55.6% while Malay student were 44.44%. A majority of the students were male students with 54.3 %. Most of the students (60.5%) were in normal weight but 25.9% from the total students, were obese

Fecal bacterial composition among primary school children

The results of microbiome were constructed from Biom file that were obtained using QIIME analysis as mentioned above. The bacteria compositions of 81 samples were determined in each taxonomic level according to read the counts of the OTUs (operational taxonomic unit) in each sample that was further classified into known 18 phylum, 34 class, 57 order, 103 families and 223 genuses. From our study population, the most abundance of bacteria composition at genus level were *Bacteroides* 23%, *Prevotella* 22% and about 11% from Family of *Lachospiraceae,* unknown genus. The rest of the type of bacteria was about 1-6 % included *Faecalibacterium, Bifodobacterium, Sutterella, Collinsella, Phascolarctobacterium,* family of *Ruminococcaceae* (unknown genus) and family of *Enterobacteriaceae* (unknown genus). The bacteria type with relative abundance of less than 1% was grouped together under others which comprised of 23 % from total bacteria type **(Figure 1a).** As shown in the pie chart, **(Figure 1b and 1c**) it showed the relative abundance of bacteria type at genus level among Malay and Chinese population. For the Malay population, the predominant types were

Bacteroides 21 %, *Prevotella* 21% and 12% from family of *Lachospiraceae* (unknown genus) while in Chinese population, it was not much different compared with Malay population which were predominant type still *Bacteroides* 24 %, *Prevotella* 22 % and 11 % from family of *Lachospiraceae* (unknown genus).

Association of socio- demographic factor and BMI between gut microbiota.

Simple and multiple logistic regression analysis was conducted to determine the asscociated factor for socio- demographic data (age, race & gender), BMI and dietary intake between type of gut microbiota either predominantly *Bacteroides* (B-Type) or predominantly *Prevotella* (P-type). We used to compare with *Bacteroides* and *Prevotella* type of bacteria in view of our study population, and the most abundance of bacteria composition at genus level were *Bacteroides* and *Prevotella* as was mentioned before. From our study, there were no significant association between socio demographic factors and type of gut microbiota that was studied as *P*-value > 0.05. By using univariate analysis (chi squere test) (**Table 2a, 2b**) showed there was a significant association between BMI and type of gut microbiota either predominantly B-Type or P-Type, (*p*-value =0.005.) . Our study showed *Bacteroides* type of gut microbiota were highly enriched in normal weight children. We found that about 77.27% of normal weight children had *Bacteroides* type of microbiota. From the multivariate analysis (multiple logistic regression), an overweight and obese person was associated negatively with B-Type of microbiota, while in P-Type, there were significant positive association for an overweight person (OR=35.404,95%CI:2.153,582.191with p=0.013) and obese (OR=16.725,95% CI:2.191,127.650 with $p = 0.007$) when other variables were adjusted.

The Association of diet with gut microbiota

Using the food frequency data, we performed multiple logistic regression analysis to corelate diet and the type of gut microbiota. **Table 3a and 3b** shows factors associated with *Bacteroides* and *Prevotella* type of gut microbiota among primary school children in Kota Bharu. The result obtained from statistical analysis, indicated that chicken (OR=6.869,CI:1.701,27.730,*p*=0.007) and fish (OR=6.760 CI:1.110,41.170,*p*=0.038)intake perday had significant positively associated with B-type, whereas fruits, seasoning and flavourings and beverages associated negatively with B-Type when other variables were controlled **(Table 3a).**While in P-Type, fruit, milk and dairy product ,seafood, seasoning and flavourings and beverage were associated positively with significant *p* -value < 0.05 when other variables were adjusted **(Table 3b).** Other types of food were not significant with either type of gut microbiota as *p*-value > 0.05.

3.6 DISCUSSIONS

The objective of the present study were to investigate the association between *Bacteroides* and *Prevotella* type of gut micobiota with socio-demographic factors (age, race and gender), BMI and diet among healthy primary school in Kota Bharu population. Our study reveals that diet and BMI has a dominant role over other possible variable such as age, ethnicity and gender in shaping the gut microbiota as our socio-demographic factors were not significant with p value > 0.05. The effect of diet modification corresponding to different socio-economic condition on gut microbial communities was not investigated in present study. De Fillipo et al (2010) , demonstrated that children living in a rural African village in Burkino Faso, an environment resembling that of Neolithic subsistence farmers, is completely different from the microbiota of children living in the urban Western world (3).

The gut microbiota varies immensely between individuals. Here we found that *Bacteroides* was the most prevalent genus in the normal weight children group, a finding that consistent with Hu et al 2015 who found that *Bacteroides* was the most prevalent genus in the normal adolescent group (16). However, these findings were inconsistent with Agans et al, who found that *Ruminococus* was the most prevalent genus in the normal adolescent group. (17). On the other hand, *Prevotella* – type of microbiota showed correlation with overweight and obese children group. This agrees with a previous paper by Durban et al 2012 and Hu et al 2015, who found that higher level of prevotella in obese subjects (16, 38), whereas in other study by Nakayama J et al 2017 had oppositely reported that low abundance of prevotella type in overweight and obese children (18)

Diet is considered a major contributing factor for changes in gut microbiota diversity as it provide nutrition and alters with microenvironment for microbes. (1) The *Prevotella* human enterotype is associated with high intake carbohydrate and simple sugar, indicating an association with carbohydrate base diet typical of agrarian societies (1, 6). The present study showed the subject who take high frequency of fruits perday which correlated positively with P-Type (OR=2.214, *p*-value 0.025), whereas it correlated negatively with B-Type. This finding was consistent with Nakayama et. al. (2017), in which they found that Leyte children who ingested β-carotene/vitamin A, mainly from regional fruits, such as green mango and banana, had positive correlation with P-Type microbiota. The regional fruits are also known to contain high amount of dietary fibers (18). This suggests that our population who take high frequency of plant-polysaccharide from fruits highly are associated with P-Type microbiota. This result can be further explained if we measured short chain fatty acid level (SCFA). As in other study plant polysaccharides producing high level of SCFA that supply the host with an additional amount of energy (3). In our study population, wheat and rice are not significantly associated with either type of microbiota as *p*-value > 0.05. This finding is inconsistent with Nakayama et. al. (2015,) as they found that rice intake frequency significantly correlated with P-type microbiota, suggesting that other factors associated with our population strongly influence on microbiota type. One possible explanation is the difference in the cultivar of rice eaten daily in other population, which differs in the fine structure of starch and influences digestion and absorption in the intestine (19).

Bacteroides enterotype has been proposed to be associated with a diet high in animal protein, a variety of amino acid and saturated fat (1, 3, 6). In the present study, chicken and fish intake frequency positively correlates with B-Type microbiota. Chicken (OR=6.869, *p*= 0.007) and fish (OR=6.760, *p*=0.038) showed higher odds ratio while in P-Type microbiota it

correlated negatively. In contrast with other studies, egg and chicken correlated positively with P-Type (15). It suggested that eggs and chickens contain high concentration of vitamin A and vitamin B5, which may support the growth of *Prevotella* in intestinal (8). This may be explained by none investigated factors, maybe including host genetic or interaction with gut microbiota.

David and collegues[7] found that microbiota changes on the animal based diet could be linked to altered fecal bile acid profile and potential for human enteric disease such as inflammatory bowel disease(8). Overall a balance of gut microbiota composition gives benefits to the host while imbalances of gut microbes were associated with metabolic and immune mediated disorder (1). Therefore, further studies are warranted to address the question of hidden factors such as host genetics which may interact with their gut microbiota.

The present study has some limitation. Inadequate sample size was one of our limitations. We need a larger sample size in order to represent of our population. The mechanism by which BMI influences *Bacteroides* or *Prevotella* level was not investigated in present study. These previousy had been investigated on microbially-derived short chain fatty acids (SCFA), which play different roles in energy salvage (11, 19). The further analysis need to be addressed to determine the difference in metabolic activity between the two types of microbiota. It appears that B-Type microbiota is well nourished and metabolically more active with simple sugars, amino acids and lipids, while in P-Type is more involved in digestion of complex carbohydrate (18).

In summary, this study revealed an association between BMI and diet on gut microbiota among healthy primary school children in Kota Bharu population. Although we found the

correlation between obesity and enterotype in this study, it is yet to be answered whether the altered gut microbiota is a cause of obesity or just a consequence of altered dietary habit. Further studies are necessary to elucidate the mechanism behind these changes and ultimately their link to health and disease.

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CONFLICT OF INTEREST DECLARATION

None declared.

3.7 REFERENCE

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3.8 TABLE AND FIGURE.

Table 1 Sociodemographic and physical characteristic of participants (n=81)

Variable	Mean (sd)	n (%)
1) Age	9.12 (1.36)	
2) Year		
Standard 1		12 (14.8%)
Standard 2		18 (22.2%)
Standard 3		15 (18.5%)
Standard 4		20 (24.7%)
Standard 5		16 (19.8%)
3) Gender		
- Male		44 (54.3%)
- Female		37 (45.7%)
4) Race		
- Malay		36 (44.44%)
- Chinese		45 (55.6%)
5) Weight	33.26 (13.08) kg	
6) Height	129.69 (11.33) cm	
7) BMI	19.18 (4.99) kg/m^2	
8) BMI category		
Underweight		3 (3.7%)
Normal weight		49 (60.5%)
Overweight		8 (9.9%)
Obesity		21 (25.9%)

Table 2a Association of BMI with *Bacteroides* type of gut microbiota among healthy primary school children

Variable	Bacteroides			
	Yes n(%)	No n(%)	X^2 stat (*df*)	*p*-value
BMI category			3	0.005
Underweight	1 (2.3%)	2 (5.4%)		
Normal weight	34(77.3%)	15(40.5%)		
Overweight	1 (2.3%)	7(18.9%)		
Obese	8(18.2%)	13(35.1%)		

Table 2b Association of BMI with *Prevotella* type of gut microbiota among healthy primary school children

Variable	Prevotella			
	Yes n(%)	No n(%)	X^2 stat (*df*)	*p*-value
BMI category			3	0.005
Underweight	2 (5.4%)	1 (2.3%)		
Normal weight	15(40.5%)	34(77.3%)		
Overweight	7 (18.9%)	1(2.3%)		
Obese	13 (35.1%)	8(18.2%)		

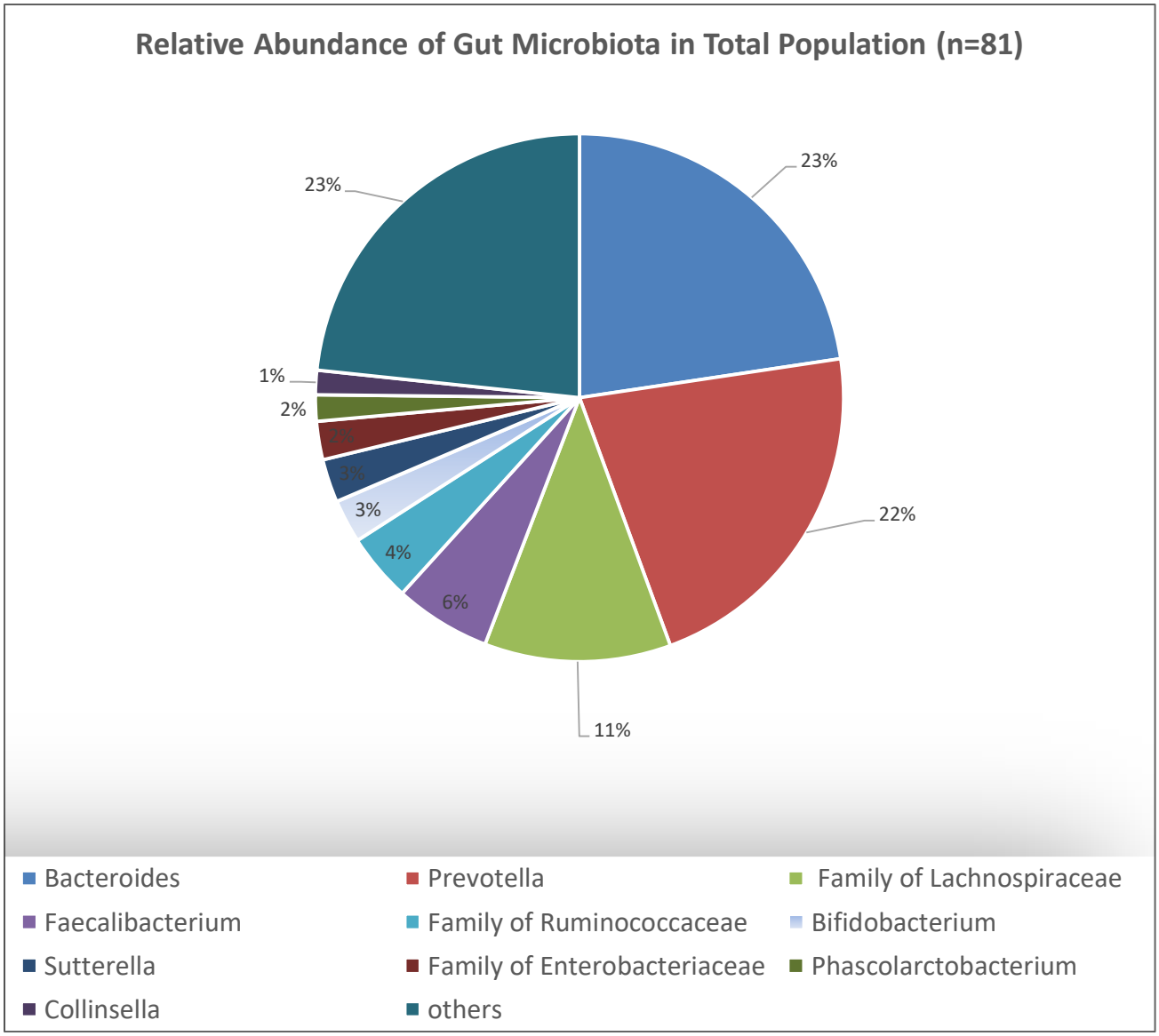


Figure 1a; Relative abundance of bacteria types from study population (n=81) among primary school children in Kota Bharu. Bacteria type with relative abundance of < 1% is grouped together under 'others'

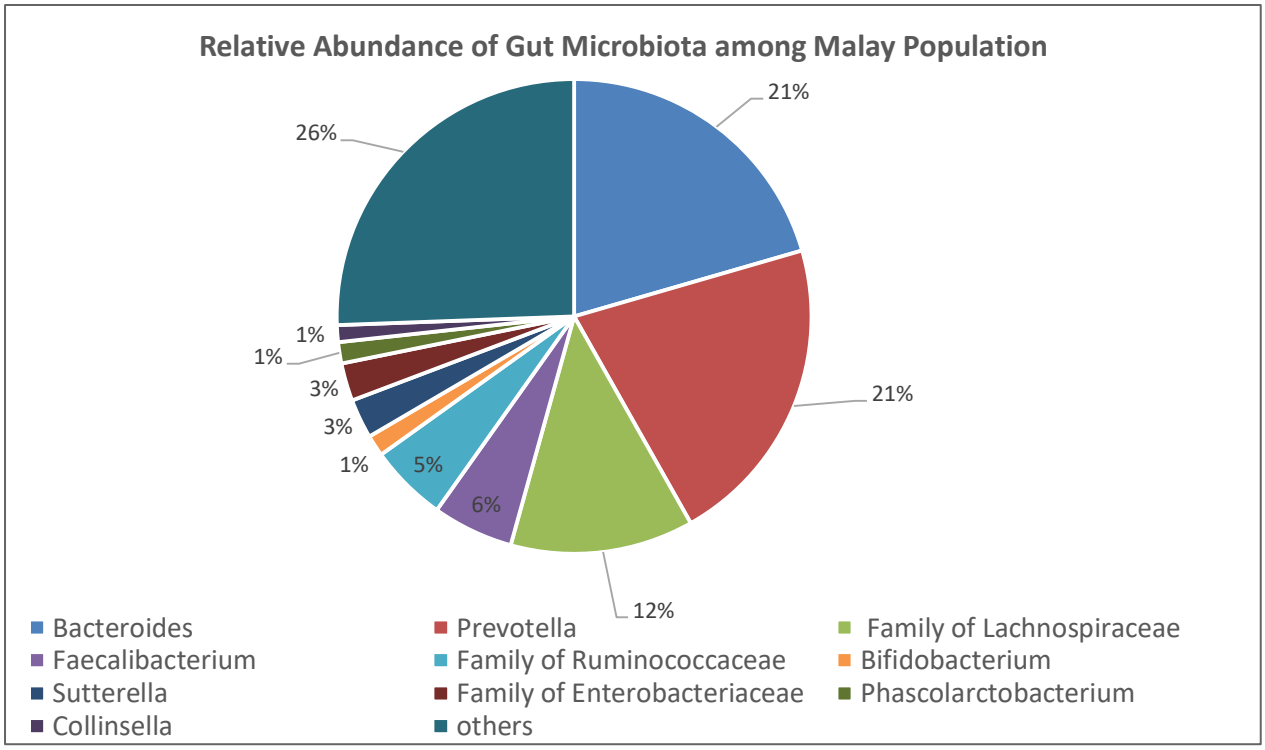


Figure 1b; Relative abundance of bacteria among Malay population (n=36). Bacteria type with relative abundance of < 1% is grouped together under 'others'

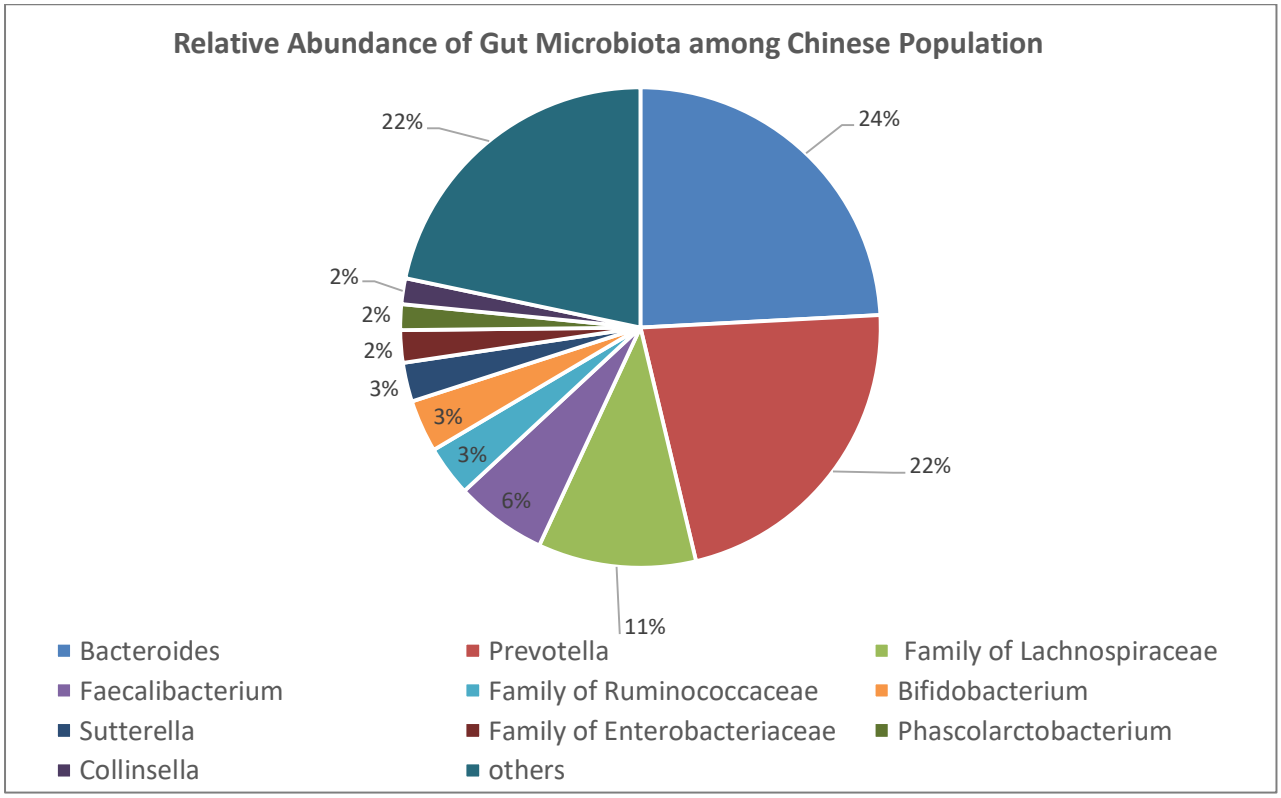


Figure 1c; Relative abundance of bacteria among Chinese population (n=45). Bacteria type with relative abundance of < 1% is grouped together under 'others'

Table 3a; Associated factors with *Bacteroides* type of bacteria among primary school children in Kota Bharu.

Variable	Simple Logistic Regression		Multiple Logistic Regression		
	Crude OR (95% CI)	p-value	B	Adjusted OR(95% CI)	p-value
BMI category					
Underweight	0.221 (0.019, 2.624)	0.232	-2.452	0.0861(0.005,1.642)	0.103
Normal weight	1	0.013			**0.022**
Overweight	0.063 (0.007,0.558)	0.013	-3.160	0.042(0.003,0.584)	0.018
Obese	0.271 (0.093, 0.791)	0.017	-2.116	0.121 (0.024, 0.612)	0.011
Dietary intake					
Wheat	0.975 (0.777, 1.224)	0.826	0.228	1.256 (0.627, 2.516)	0.520
Rice	1.079 (0.898, 1.295)	0.417	0.092	1.096 (0.769, 1.561)	0.612
Chicken	1.539 (0.892, 2.655)	0.122	1.927	6.869 (1.701, 27.730)	**0.007**
Fish	0.968 (0.707, 1.327)	0.842	1.911	6.760 (1.110,41.170)	**0.038**
Fruits	0.828 (0.599, 1.147)	0.256	-0.771	0.463 (0.242, 0.883)	0.019
Seasoning and flavourings	0.800 (0.644, 0.994)	0.044	-0.780	0.458 (0.277, 0.758)	0.002
Beverages	0.627 (0.427, 0.921)	0.017	-0.793	0.452 (0.240, 0.851)	0.014

Constant= 2.852
Backward LR Method was applied
No multicollinerity and no interaction
Hosmer Lemeshow test, P value = 0.604
Classification table 82.7 % correctly classified
Area under Receiver Operating (ROC) curve was 90.2 %

Table 3b: Associated factors with *Prevotella* type of bacteria among primary school children in Kota Bharu.

Variable	Simple Logistic Regression		Multiple Logistic Regression		
	Crude OR (95% CI)	P value	B	Adjusted OR(95% CI)	P value
BMI					
Underweight	4.533 (0.381,53.925)	0.232	3.473	32.227 (1.385,749.715)	0.031,
Normal weight	1	0.013			**0.016**
Overweight	15.867 (1.791,140.584)	0.013	3.567	35.404 (2.153,582.191)	**0.013**
Obese	3.683 (1.263,10.738)	0.017	2.817	16.725 (2.191, 127.650)	**0.007**
Dietary intake					
Wheat	1.026 (0.817, 1.288)	0.826	-0.602	0.547(0.252,1.187)	0.127
Chicken	0.650 (0.377,1.121)	0.122	-2.498	0.082(0.016, 0.431)	0.003
Fish	1.033 (0.754, 1.415)	0.842	- 2.498	0.082 (0.011, 0.616)	0.015
Seafood	1.111 (0.897,1.376)	0.335	1.984	7.273(1.189,44.505)	**0.032**
Fruits	1.207 (0.872, 1.670)	0.256	0.795	2.214 (1.104, 4.441)	**0.025**
Milk and dairy product	1.182 (0.917, 1.525)	0.197	0.718	2.050 (1.118, 3.757)	**0.020**
Spread dressing	0.975 (0.745,1.276)	0.852	-0.581	0.559 (0.313, 0.999)	0.049
Seasoning and flavourings	1.250 (1.006, 1.553)	0.044	1.007	2.737 (1.497, 5.003)	**0.001**
Beverages	1.595 (1.085,2.343)	0.017	0.919	2.506 (1.223,5.136)	**0.012**

Constant= - 4.132
Backward LR Method was applied
No multicollinerity and no interaction
Hosmer Lemeshow test, P value = 0.481
Classification table 85.2% correctly classified
Area under Receiver Operating (ROC) curve was 92.3%

CHAPTER 4

STUDY PROTOCOL SUBMITTED FOR ETHICAL APPROVAL

ROLE OF DIET AND BMI ON BASAL GUT MICROBIOTA PROFILE IN A PRIMARY SCHOOL IN KOTA BHARU.

1)INTRODUCTION

Normal gut microbiota is essential for digestive processes such as fermentation of carbohydrates, but it also takes part in immunological processes by participating in the development of gut-associated lymphoid tissues (GALTs) and providing resistance to pathogens [1]. Dysbiosis of gut microbiota has been linked to several metabolic and immunological disorders such as diabetes, irritable bowel syndrome, and allergies [1].

Determining what constitutes a healthy microbiota and the variability found across populations is a prerequisite for assessing deviations that are associated with disease states.[2].The gut microbiota is typically dominated by bacteria and specifically by members of the divisions Bacteroidetes and Firmicutes (Turnbaugh et al., 2006). [2]

Dietary changes in particular have been shown to have significant effects on the microbiota.it has been shown in mice that shifting to a high-fat, high-sugar ‘‘Western’’ diet from a low-fat,plant polysaccharide-rich diet can change the microbiota within a day (Turnbaugh et al., 2009b) In another study in humans,shifting from a high-fat/low-fiber diet to a low-fat/high-fiber diet caused notable changes in the gut microbiota within 24 hr (Wu et al., 2011). [3,] diet also correlates with enterotype, as individuals on a diet high in animal fat have a Bacteroides- dominated enterotype, whereas a carbohydrate-rich diet is associated with the Prevotella-dominated enterotype (Wu et al., 2011).[3]

So far, several studies in humans and mice have shown differences in gut microbiota composition between obese and lean subjects. These differences were mostly detected at the phylum level of mainly Firmicutes and Bacteroidetes [4-7]. Obesity in humans has already been associated with low intestinal concentrations of Bacteroidetes and high concentrations of Firmicutes, although this finding has been contradicted by other studies [8,9]. Only few studies have investigated the prevalence of faecal bacterial phyla in obese children and adolescents. All these studies mentioned were conducted in the west, with very different dietary and cultural habit from those of Asia, In the present proposal, we plan to conduct the study to examine the microbiota profile among healthy youngster in our country as the pilot study to provide the background for further perspective studies of disease population and age groups.

2) OBJECTIVE

GENERAL OBJECTIVE

1) To determine the basal Gut Microbiota profile among healthy primary school children of 7-11 years in Kota Bharu, Kelantan

SPECIFIC OBJECTIVE

1) To determine association of demographic data (age, gender and race) between *Bacteroides* and *Prevotella* among healthy primary school children
2) To determine association of BMI between *Bacteroides* and *Prevotella* among healthy primary school children after controlling the effect of subject demographic and diet.
3) To determine association of dietary intake between *Bacteroides* and *Prevotella* among healthy primary school children after controlling the effect of subject demographic and BMI.

3) ALTERNATIVE HYPOTHESIS

1) There are association between *bacteroides* and *Prevotella* with BMI and dietary habit among healthy primary school children

4)METHODOLOGY

I. Study design and period

- 1**)** Study design **:** cross sectional study
- 2) Subject recruitment period for data analyses : 1st March 2016 – 31th August 2017.
- 3) Study location**:** Primary schools, Kota Bharu, Kelantan, Malaysia

II. Sampling population

- Base on State Education Department database, 6 primary schools will be selected, which involved 3 Malay schools and 3 Chinese schools.
- The age group of the students is selected between 7-11 years old.
- Base on approval from ministry of education, they not allow 12 years old student who are going for UPSR examination to be involved in this study.

III. Study population

- Healthy volunteers will be screened from medical record from each school that involved and 82 subjects will be recruited after informed consent from parents and child.
- This age group of subjects is selected with the reason that children aged between 7-11 years old are largely living at home and consuming home cooked food.
- All participants will have their gender, ethnic group, dietary habits, weight, height and health condition documented in a questionnaire

IV. Fecal sampling processing

- The fecal samples will be collected one time at the household level by each participating child with the help of their parents/guardian.Technique for fecal collection is shown in figure 1 below.
- For fecal collection, we provide the child with chinese paper covered by yellow plastic and float that yellow plastic on water surface in a lavatory bowl. Fecal specimens should not be contaminated with water or urine.
- A portion of freshly-voided feces will be collected into the sampling tube, and then suspended into RNAlater reagent (the reagent for stabilizing the nucleic acid).
- The tube will be shaken vigorously for 10 seconds to suspend the feces in the solution. The tube will be stored at room temperature (avoiding sunshine or higher temperature) and must be sent back to the investigator team within 7 days after collection.
- Once stool samples are available, a person in-charged from the school will notify us to visit the school for the sample collection.
- Once the samples have been collected, the sample will be sent to our laboratory for immediate storage and for subsequent processes.
- In the laboratory, the fecal samples will be processed for nucleic acids extractions, which will be stored at -80 degree celcius until use. DNA and RNA will be extracted from the fecal samples.
- Pyrosequencing will be used to examine gastrointestinal microbiota profile in fecal samples. 16S rDNA V6-V8 segment will be amplified from fecal DNA extracts by PCR using eubacterial universal primers with bar-code tag sequence allowing sample assignment after the one batch multisample sequencings.
- The amplicons will be subjected to pyrosequencing and the resultant sequences of more than 1,000 reads expected for one sample will be subjected to database search to find closest taxon.
- The taxonomic information of each read will be summed to gain bacterial composition of each sample.
- The population data is expected to cover any group of bacteria containing uncultured or unknown species with their detection level corresponding to approximately more than 0.1% of total population.

1. Preparation of Fecal Sample

1.1. Fecal Sampling (by the donor)

Collect the fecal sample according to the following procedure.

Materials

- Fresh feces
- Fecal collection tube
- Glass beads (ø 2.5 mm)
- RNA*later*®
- Trail paper

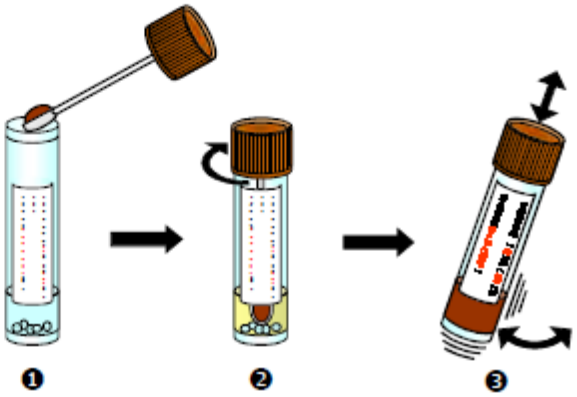
➢ Fecal Collection Tube..... Take 2 ml RNA*later*® and 5 ~ 10 glass beads (ø 2.5 mm) in a tube, and measure the weight prior to fecal sampling.

Procedure

1) Float a trail paper on water surface in a lavatory bowl. Refer to Picture 1.
2) Using the spatula attached to the tube cap, take one spatula portion (0.3 ~ 0.5 g) of the feces defecated in the trail paper. Avoid taking feces dipped with urine or water (Fig. 1❶).
3) Put the spatula into the fecal collection tube, and cap the tube tightly (Fig. 1❷).
4) Shake the tube vigorously for 10 seconds to suspend the feces in RNA*later*® (Fig. 1❸).
5) Store it at 4 ˚C within 3 weeks. This series of steps have to be performed as soon as possible after the defecation.

Picture 1

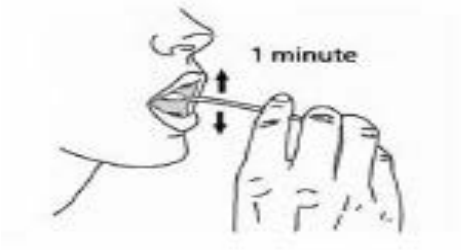
Fig. 1. Fecal sampling



V. Buccal swab

- For determination of oral microbiota and that possible correlation between gut microbiota.
- we only measured microbiota genom, no human genom will be analysed.
- Estimated time taken for buccal swab procedure, took not more then 5 minutes.

- 1. Fill in volunteer details on the form and label provided.
- 2. Before obtaining a mouth swab, rinse mouth with water twice.
- 3. Unseal the package to collect the mouth swab. With a firm grip, unscrew mouth swab from its plastic tube. ***NOTE: Do not touch the swab tip with your hands!***
- 4. Swallow saliva before you swab.
- 5. Brush swab firmly against inside of your RIGHT cheek for 1 minutes. Cover as much area of whole cheek as possible. Rotate swab as you brush. Repeat collection with your LEFT cheek.
- 6. Insert swab back into its plastic tube and press firmly to seal the tube.
- 7. Stick the label unto the plastic tube, store the mouth swab in a sealed envelope labelled with the volunteer ID and keep in 4°C or -20°C freezer



Patient code: ☐☐☐☐ **Gender:** Male / Female

Ethnicity: Chinese / Malay / Indian / _____

Age: _____ **Country of origin:** _____

Collected by: _____ (NAME/INITIALS) **Date:** ___ / ___ / ___ (DD / MM / YY)

Please paste sticker on tubes.

VI. Destruction of specimen at the end of study.

- Residual samples of buccal swab and stool will be discarded

VII. Sample size calculation

The estimated sample size this study calculated using PS Software.

Objective 1: Was calculated using PS software using two proportion (independent) formula. The estimated sample size was 82 with 80% power, 5% significance level, 10% drop-out rate (assuming P0 = 0.2 , P1= 0.5, M = 1) (Hu, H.-J., et al. 2015).

Objective 2 : Was calculated using PS software using two proportion (independent) formula. The estimated sample size was 71 with 80% power, 5% significance level, 10% drop-out rate (assuming P0 = 0.2 , P1= 0.6 , M= 4) (Nagwa et al,2010)

Objective 3 : Was calculated using PS software using two proportion (independent) formula. The estimated sample size was 70 with 80% power, 5% significance level, 10% drop-out rate (assuming P0 = 0.1 , P1= 0.4 , M= 1) (Wu, G. D., et al. 2011)

As conclusion, the estimated sample size was 82 that were calculated for objective 1 was chosen for this study because it was the largest sample size calculated.

VIII. Data & statistical analysis.

- Acquisition of data is made using the provided analytical software.
- All data will then be entered and analyzed using the SPSS software (version 21, SPSS Inc., Chicago, IL, USA).
- The numerical data would be expressed in mean (standard deviation, SD) with categorical in frequency and percentages.
- The Chi-square test used to test whether gender showed an equal distribution between normal and obese group. Age, height, weight, and BMI were compared using student's t-test. The association between microbiota composition and BMI was expressed in terms of pearson's correlation coefficient. [11]
- Comparison in continuous outcomes between study groups will be made using Anova test whereas Chi Square test will be used for comparing between different category variable. A p value of <0.05 is considered significant.
- For dietary evaluation, were evaluated according to the food pyramid guidelines. Food frequency questionnaires (FFQs) ask about the usual frequency of consumption of a list of foods mainly to evaluate fat, protein and carbohydrate intake. [12]

5) DURATION OF HUMAN SUBJECT INVOLVEMENT

3 visits over 6 week's period

- First visit: healthy volunteers will be screen from medical record in each school, if the children agree to participate in this study; their parents will be contacted to obtain their agreement to allow their children to participate in the study. In addition, the parent's consent form will be sent to the parents by their children
- At second visit, for those who are consented to participate in this study, the children will be asked to provide information about their medical history and

dietary history. The parents will also be contacted via phone and a meeting among investigators team and parents will be made in order to get further information about the children's health, dietary habit and the way to collect the buccal and stool sample

- Once the buccal and stool samples are available, a person in-charged from the school will notify us to visit the school for the sample collection. Once the samples have been collected, they will be sent to our laboratory for immediate storage and for subsequent processes.
- Estimated time taken for each procedure (both buccal and stool sampling), its took about not more then 5 minutes.

6) INCLUSION CRITERIA

1) Age between 7-11 years old

2) Parents and child able to provide consent for providing stool sample and buccal swab sample.

7) EXCLUSION CRITERIA

1) Suffering from a chronic medical and surgical condition.
   Chronic medical illness: eg diabetes, heart failure, inflammatory bowel disease, irritable bowel syndrome or any disease related to gastrointestinal disease.
   Surgical illness: post bowel surgery (on colostomy bag), or any disease that related to gastrointestinal disease.
2) Any recent illness (3 months) that may compromise the immune system, eg: Pneumonia, Acute gastroenteritis or any febrile illness.
3) Have been taking probiotic/prebiotic product diets such as yogurt or cheese from 2 weeks before sampling.
4) History of taking antibiotic within 3 months
5) Taking outside food > 50% of meals.(took food that not prepared at home more than 10 meals in a week)
   # In normal circumstance, we took average 21 main meals (7x3) in a week.

8) WITHDRAWAL CRITERIA

1) The child not able to follow the instruction during procedure.
2) The child getting sick during research period, e.g.: having acute illness/febrile illness that required antibiotic treatment or developed acute gastroenteritis.

9) VULNERABILITY OF THE SUBJECT

- This study involves children as the study subjects; the folowing must be obliged;

i) Seeking parental permission for the child to participate in the research. Consent from parents/guardian will be recruiting via meeting among researcher and parents. Besides

consent, detail diet history, instruction for stool sampling and guidance for answering questionnaire will be obtained during meeting with parents/guardian.

ii) Securing the child's assent to participate in the research. Verbal consent will be getting from the child below 10 years, wheres more than 10 years, they need to sign the simplified assent form.

10) THE IMPORTANCE AND BENEFITS OF THE RESEARCH:

The profile of basal microbiota obtained from this study will be used as reference for all our subsequent clinical studies in Kelantan.
The same data will also be serve as reference by other centres in Malaysia since there are no such data previously.
Having these normative data will also serve as benchmark for any clinical trials that are enrolling Malaysian centres

11) HANDLING PRIVACY & CONFIDENTIALLITY ISSUE

- The medical information of each subject will be kept confidential and will not be made publicly available unless disclosure is required by law.
- The data obtained from this study that does not identify the subject will be published for knowledge purposes.
- the subject medical information may be held and processed on a computer.

12) INCENTIVES/HONORARIUM/COMPENSATION

- For subject's participant, each subject will be reimbursed with honorarium for the time that being contributed.

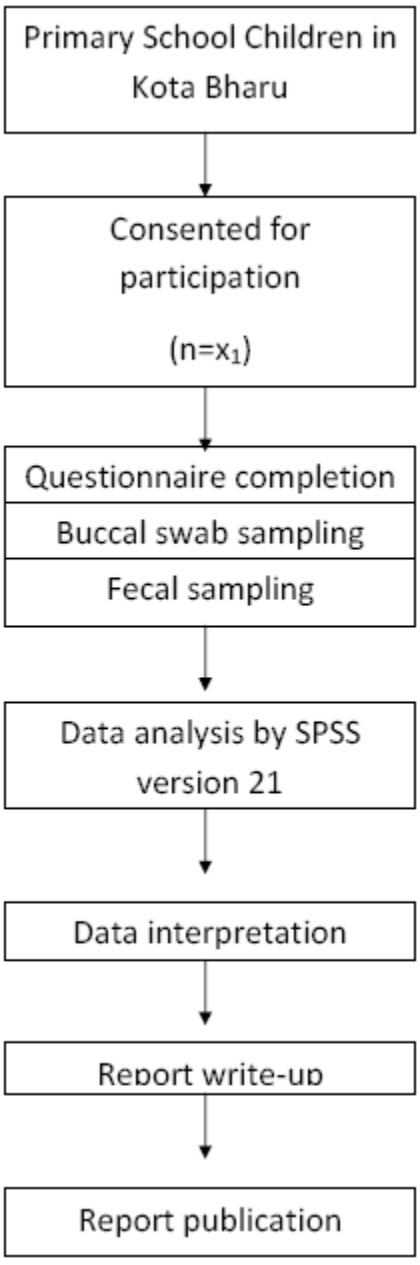
13) OPERATIVE DEFINITION

I. The children were classified as obese or normal-weight, based on gender and age-specified BMI percentiles from the Centers for Disease Control (http://www.cdc.gov), in which obesity is defined as $\geq 95^{th}$ percentile and normal-weight defined as < 85^{th}persentile.
II. Chronic medical illness: eg diabetes, inflammatory bowel disease.
III. Surgical conditions: eg, post bowel surgery (on colostomy bag)
IV. Outside food: food not prepared at home

EXPECTED RESULT

	GUT MICROBIOTA	
	Bacteroides type	*Prevotella* type
	Odds ratio	P- value
1) Age		
2) Gender -male - female		
3)BMI -Obese - Normal weight		
4) Dietary - Carbohydrate - Protein - Fat		

STUDY FLOW CHART



GANNT CHART

YEAR	2015						2016												2017					
MONTH	JUL	AUG	SEP	OCT	NOV	DEC	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	JAN	FEB	MAR	APR	MAY	JUN
Ethical approval (USM level)					→	→	→																	
Preparation of study material								→	→	→														
Data collection										→	→	→	→											
Data analysis and interpretation													→	→	→	→	→	→						
Report writing																			→	→	→	→	→	
Report publication																							→	→

Planned milestones

1) Ethical approval : November 2015→ February 2016
2) Preparation for study material : February 2016→March 2016
3) Data collection : March 2016→ Jun 2016
4) Data analysis and interpretation : July 2016→ October 20 16
5) Report writing : November 2016→ May 2017
6) Report publication : June 2017→ August 2017

4.2 Patient information and consent form

ATTACHMENT B

PARENTAL INFORMATION AND CONSENT FORM

RESEARCH INFORMATION

Research Title: **Role of Diet and BMI on Basal gut microbiota profile in a primary school children in Kota Bharu, Kelantan**

Researcher's Name: **Dr Nur Amalina Bt Muhammad (MMC No : 47693)**

Co-investigators:

1) A Prof Lee Yeong Yeh (MMC No : 36810)
2) A Prof Norizan binti H A Majid (MMC No:26234)
3) A Prof Siti Asma binti Hassan (MMC No;34686)
4) A Prof Chan Yean-Yean
5) A Prof Lee Yuan-Kun
6) Dr Wong Mung Seong (MMC No: 45441)
7) Dr Wong Pak Kai (MMC No : 45546)

INTRODUCTION

Your child are invited to participate in a research study that involves voluntary assessment related to diet and basal microbiota of the intestine. The evaluation involves the record of the dietary intake and its relationship with the intestinal microbiota will be done periodically over six months period. Before agreeing to participate in this research study, it is important that you read and understand this form. If your child participate, you will receive a copy of this form to keep for your record.

Your child participation in this study is expected to last up to 6 weeks. Up to 66 subjects will be participating in this study. Permission from Ministry of ducation and School already approved before this research started.

PURPOSE OF THE STUDY

The purpose of this study is to determine the relationship between the dietary habit and the basal microbiota of the intestine among primary school children in Kota Bharu, Kelantan.

There is a possibility that the information collected during this study will be analysed by the researcher in the future to evaluate the relationship between diet and basal microbiora profile for other medical or scientific purposes other than those currently proposed.

QUALIFICATION TO PARTICIPATE

The doctor in charge of this study or a member of the study staff has discussed with you the requirements for participation in this study. It is important that you are completely truthful with the doctor and staff about your child health history. Your child should not participate in this study if your child do not meet all qualifications.

Some of the requirements to be in this study are:

- Age between 7-11 years old
- Parents able to provide consent for providing stool sample and buccal mucosa.

Your child cannot participate in this study if:

- Your child are suffering from a chronic medical and surgical conditon such as diabetis mellitus, inflammatory bowel disease and any bowel surgery
- Your child have any recent illness (3 months) that may compromise the immune system.eg: chest infection or acute gastroenteritis.
- Your child have been taking probiotic/ prebiotic product diets such as yogurt or cheese from 2 weeks before sampling.
- History of taking antibiotic within 3 months
- Taking outside food > 50 % of meals (took food that not prepared at home more than 10 meals in a week)
  # In normal circumstance, we took average 21 main meals (7x3) in a week.

STUDY PROCEDURES

At first visit, if the children agree to participate in this study, their parents will be contacted to obtain their agreement to allow their children to participate in the study. In addition, the parent's consent form will be sent to the parents by their children.

At second visit, for those who are consented to participate in this study, the children will be asked to provide information about their medical history and dietary history. In addition, they will be given the instruction about the diet, the way to collect buccal and stool sample. The parents will also be contacted via phone and a meeting among doctor and parents will be made in order to get further information about the children's health, dietary habit and the way to collect the buccal and stool sample

Once the buccal and stool samples are available, a person in-charged from the school will notify us to visit the school for the sample collection. Once the samples have been collected, they will be sent to our laboratory for immediate storage and for subsequent processes.

RISKS

During the process of buccal swap sampling, there might be some minor injury during the procedure. As for fecal sampling, this procedure involves no risk during the process of sampling.

REPORTING HEALTH EXPERIENCES.

If you have any injury, bad effect, or any other unusual health experience during this study, make sure that you immediately inform Dr. Nur Amalina Bt Muhammad [MMC Registration No.47693]at 09-767 6590/ 012-9049947

PARTICIPATION IN THE STUDY

Your child taking part in this study is entirely voluntary. Your child may refuse to take part in the study or your child may stop participation in the study at anytime, without a penalty or loss of benefits to which your child are otherwise entitled.For your child participation, we will imburse your child some money for your child time constributed. Your child participation also may be stopped by the study doctor or sponsor without your consent.

The buccal and stool samples will only be analysed according to tests/ procedures mentioned in the protocol and not beyond.Any residual samples of buccal swab and stool will be discarded.

This current research will not arrive at any disease/ diagnosis at the moment, and the information gathered will only be informed upon formal request.

POSSIBLE BENEFITS [Benefit to Individual, Community, University]

With the information obtained, the profile of basalgut microbiota will be used as reference for all our subsequent clinical studies in Kelantan.

The same data will also be serve as reference by other centres in Malaysia since there are no such data previously.

Having these normative data will also serve as benchmark for any clinical trials that are enrolling Malaysian centres.

QUESTIONS

If you have any question about this study or your rights, please contact;

Dr Nur Amalina Bt Muhammad & MMC No. 47693
Department of Internal Medicine
PPSP, USM Health Campus
Tel: 09-767 6590/ 012 9049947

If you have any questions regarding the Ethical Approval or any issue / problem related to this study, please contact;

Mr. Mohd Bazlan Hafidz Mukrim
Secretary of Human Research Ethics CommitteeUSM
Centre for Research Initiatives, Clinical & Health Sciences
USM Health Campus
Tel. No. : 09-767 2354 / 09-767 2362
Email : bazlan@usm.my/jepem@usm.my

CONFIDENTIALITY

Your child medical information will be kept confidential by the study doctor and staff and will not be made publicly available unless disclosure is required by law.

Data obtained from this study that does not identify your child individually will be published for knowledge purposes.

Your original medical records may be reviewed by the researcher, the Ethical Review Board for this study, and regulatory authorities for the purpose of verifying clinical trial procedures and/or data. Your child medical information may be held and processed on a computer.

By signing this consent form, you authorize the record review, information storage and data transfer described above.

SIGNATURES

For your child to be entered into the study, you or a legal representative must sign and data the signature page [ATTACHMENT S and ATTACHMENT P]

Parents/ Information and Consent Form
(Signature Page)

Research Title: **Role of Diet and BMI onBasal gut microbiota profile in a primary school children in Kota Bharu, Kelantan**

Researcher's Name **:** **Dr Nur AmalinaBt Muhammad(MMC 47693), Dr Wong Mung Seong(MMC45441), AP Lee YeongYeh(MMC 36810), AP NorizanBintiH.A.Majid(MMC 26234), AP SitiAsmaBintiHassan(MMC 34686),AP Chan Yean-Yean, AP Lee Yuan-Kun, Dr Wong Pak Kai(MMC 45546)**

For your child to become a part this study, you or legal representative must sign this page.By signing this page, I am confirming the following:

- I have read all of the information in this Patient Information and Consent Form including any information regarding the risk in this study and I have had time to think about it.
- All of my questions have been answered to my satisfaction.
- I voluntarily agree my child to be part of this research study, to follow the study procedures, and to provide necessary information to the doctor, nurses, or other staff members, as requested.
- I may freely choose to stop my child being a part of this study at anytime.
- I have received a copy of this Parents Information and Consent Form to keep for myself.

Parent's/Guardian Name	Student's Name
Parent's/Guardian I.C No.(New)	Student's I.C No.
Signature of Parents or Legal Representative	Date (dd/MM/yy) (Add time if applicable)
Name of Individual ConductingConsent Discussion	
Signature of Individual Conducting Consent Discussion	Date (dd/MM/yy)
Name & Signature of Witness	Date (dd/MM/yy)

Note: i) All subject/patients who are involved in this study will not be covered by insurance.

ATTACHMENT G

Patient/Subject Information and Consent Form
(Signature Page)

Research Title: **Role of Diet and BMI onBasal gut microbiota profile in a primary school children in Kota Bharu, Kelantan**

Researcher's Name: **: Dr Nur Amalina bt Muhammad (MMC47693)Dr Wong Mung Seong(MMC45441), AP Lee YeongYeh(MMC36810), AP NorizanBintiH.A.Majid(MMC26234), AP SitiAsmaBintiHassan(MMC 34686),AP Chan Yean-Yean, AP Lee Yuan-Kun, Dr Wong Pak Kai(MMC45546)**

For your child to become a part this study, you or legal representative must sign this page. By signing this page, I am confirming the following:

- I have read all of the information in this Patient Information and Consent Form including any information regarding the risk in this study and I have had time to think about it.
- All of my questions have been answered to my satisfaction.
- I voluntarily agree my child to be part of this research study, to follow the study procedures, and to provide necessary information to the doctor, nurses, or other staff members, as requested.
- I may freely choose to stop my child being a part of this study at anytime.
- I have received a copy of this Parents Information and Consent Form to keep for myself.

Parents Name (Print or type)	Parents Initials and Number
Parents I.C No. (New)	Parents I.C No. (Old)
Signature of parents or Legal Representative	Date (dd/MM/yy) (Add time if applicable)
Name of Individual conducting Consent Discussion (Print or Type)	
Signature of Individual Conducting Consent Discussion	Date (dd/MM/yy)
Name & Signature of Witness	Date (dd/MM/yy)

Note: i) All subject/patients who are involved in this study will not be covered by insurance.
ii) Excess samples from this research will not be used for other reasons and will be destroyed with the consent from the Research Ethics Committee(Human), USM.

ATTACHMENT P

Student's Material Publication Consent Form
Signature Page

Research Title: Basal gut microbiota profile of primary school children in Kota Bharu, Kelantan

Researcher's Name: : Dr Nur Amalina Bt Muhammad (MPM 47693), Dr Wong Mung Seong (MPM 45441) , AP Lee YeongYeh(36810), AP NorizanBintiH.A.Majid (MPM26234), AP SitiAsmaBintiHassan (MPM34686),AP Chan Yean-Yean, AP Lee Yuan-Kun, Dr Wong Pak Kai(MPM45546)

For your child to become a part this study, you or a legal representative must sign this page.

By signing this page, I am confirming the following:

- I understood that my child's name will not appear on the materials published and there have been efforts to make sure that the privacy of my child's name is kept confidential although the confidentiality is not completely guaranteed due to unexpected circumstances.
- I have read the materials or general description of what the material contains and reviewed all photographs and figures in which my child is included that could be published.
- I have been offered the opportunity to read the manuscript and to see all materials in which my child is included, but have waived my right to do so.
- All the published materials will be shared among the medical practitioners, scientists and journalist world wide.
- The materials will also be used in local publications, book publications and accessed by many local and international doctors world wide.
- I hereby agree and allow the materials to be used in other publications required by other publishers with these conditions:
- The materials will not be used as advertisement purposes nor as packaging materials.
- The materials will not be used out of contex – i.e.: Sample pictures will not be used in an article which is unrelated subject to the picture.

Parent's/guardian's Name		Student's Name
Parents I.C No.	Parent's Signature	Date (dd/MM/yy)
Name and Signature of Individual Conducting Consent Discussion		Date (dd/MM/yy)

Note: i) All subject/patients who are involved in this study will not be covered by insurance.

LAMPIRAN A

MAKLUMAT PENYELIDIKAN & KEBENARAN IBUBAPA/PENJAGA

Tajuk kajian: Peranan pemakanan dan BMI pada profil organisma usus dalam kalangan kanak-kanak sekolah rendah di Kota Bharu, Kelantan

Nama penyelidik: Dr Nur Amalina Bt Muhammad (MMC No: 47693)

Penyelidik bersama:

1) PM Lee Yeong Yeh (MMC No: 36810)
2) PM Norizan binti H A Majid (MMC No: 26234)
3) PM Siti Asma binti Hassan (MMC No : 34686)
4) PM Chan Yean-Yean
5) PM Lee Yuan-Kun
6) Dr Wong Mung Seong (MMC No: 45441)
7) Dr Wong Pak Kai (MMC No: 45546)

PENGENALAN

Anak anda dijemput untuk mengambil bahagian dalam kajian penyelidikan yang melibatkan penilaian sukarela berkaitan dengan pemakanan dan organisma dalam usus. Penilaian melibatkan rekod pengambilan makanan dan hubungkait dengan organisma usus yang akan dilakukan secara berkala dalam tempoh masa 6 bulan. Sebelum mempersetujui untuk mengambil bahagian dalam kajian penyelidikan ini, adalah penting untuk anda membaca dan memahami borang ini.Sekiranya anak anda mengambil bahagian, anda akan menerima satu salinan borang ini untuk simpanan peribadi.

Penglibatan anak anda dalam kajian ini dijangkakan hanya untuk 6 minggu.Hampir 66 orang subjek akan mengambil bahagian dalam kajian ini.Kebenaran dari Kementerian Pendidikan Malaysia dan sekolah telah diluluskan sebelum kajian ini dijalankan.

TUJUAN KAJIAN

Untuk menentukan perkaitan di antara amalan pemakanan dengan organisma usus dalam kalangan pelajar sekolah rendah di Kota Bharu, Kelantan.

Terdapat kemungkinan bahawa maklumat yang dikumpul dalam kajian ini akan dianalisa oleh penyelidik pada masa hadapan untuk menilai perhubungan di antara pemakanan dan

organisma usus untuk kajian perubatan atau tujuan saintifik lain selain daripada yang telah dicadangkan.

KELAYAKAN UNTUK PENYERTAAN

Doktor yang bertugas dalam kajian ini atau ahli kakitangan kajian sudah membincangkan dengan anak anda tentang keperluan mengambil bahagian dalam kajian ini. Adalah penting untuk anda bersikap jujur dengan doktor dan kakitangan tentang sejarah kesihatan lampau anak anda. Anak anda tidak boleh mengambil bahagian dalam kajian ini sekiranya anak anda tidak memenuhi kelayakan.

Di antara keperluan kajian dalam mengambil bahagian dalam kajian ini adalah:

- Berumur di antara 7-11 tahun
- Mendapat kebenaran daripada ibubapa/penjaga untuk menyediakan sampel najis dan calitan permukaan mulut.

Anak anda **tidak boleh** mengambil bahagian dalam kajian ini sekiranya anak anda:

- Mengalami penyakit perubatan dan pembedahan kronik, seperti kencing manis, keradangan usus yang kronik dan pembedahan yang melibatkan usus.
- Mengalami penyakit (3 bulan kebelakangan) yang akan membahayakan sistem imun, seperti jangkitan kuman di paru-paru, jangkitan kuman di usus yang menyebabkan cirit birit.
- Mengambil probiotik/produk makanan prebiotic seperti yoghurt atau keju 2 minggu sebelum persampelan
- Mengambil antibiotik dalam masa 3 bulan sebelum kajian dijalankan
- Mengambil makanan luar melebihi 50 % daripara jumlah makanan yang diambil seharian (makanan yang tidak disediakan di rumah).

PROSEDUR KAJIAN

Pada lawatan pertama,sekiranya kanak-kanak bersetuju untuk mengambil bahagian dalam kajian ini, ibu bapa akan dihubungi untuk mendapatkan persetujuan mereka membenarkan anak-anak mereka mengambil bahagian dalam kajian ini. Tambahan lagi, borang persetujuan ibu bapa akan dihantar kepada ibu bapa oleh kanak-kanak.

Pada lawatan kedua, kepada mereka yang bersetuju untuk mengambil bahagian dalam kajian ini, kanak-kanak akan diminta memberi maklumat mengenai keadaan perubatan dan sejarah

pemakanan mereka. Tambahan lagi, mereka akan diberi arahan mengenai pemakanan, cara calitan permukaan mulut dan najis. Ibu bapa akan dihubungi melalui telefon dan satu sesi perjumpaan antara doktor dengan ibubapa akan diadakan untuk mendapatkan maklumat mengenai keadaan kesihatan anak-anak mereka, tabiat pemakanan dan cara calitan permukaan mulut dan najis.

Apabila sampel permukaan dan najis sudah dikumpulkan, seorang staff yang bertanggungjawab dari sekolah akan memaklumkan kepada kami untuk hadir ke sekolah dan mengambil sampel untuk dikumpulkan. Sebaik sahaja sampel telah dikumpulkan, ianya akan dihantar ke makmal kami untuk penyimpanan segera dan pemprosesan seterusnya.

RISIKO-RISIKO

Sewaktu dalam proses pengumpulan sampel, kemungkinan akan berlaku kecederaan kecil dalam prosedur melalui calitan. Untuk sampel najis, prosedur ini tidak melibatkan risiko semasa proses pengambilan najis.

MELAPORKAN PENGALAMAN KESIHATAN

Sekiranya anda mengalami kecederaan, kesan buruk, atau mana-mana pengalaman kesihatan luarbiasa semasa kajian ini, pastikan anda memberitahu jururawat dengan kadar segera atau Dr Nur Amalina Bt Muhammad (No. pendaftaran MMC: 47693) dan no. telefon 09 7676590/0129049947

PENGLIBATAN DALAM KAJIAN INI

Anak anda mengambil bahagian dalam kajian ini secara sukarela.Anda dan anak anda boleh menolak daripada mengambil bahagian dalam kajian ini atau menghentikan penglibatan dalam kajian ini pada bila-bila masa, tanpa dikenakan penalti atau kehilangan faedah yang mana anak anda layak mendapatkannya.Penglibatan anak anda juga boleh dihentikan oleh doktor pengkaji atau penaja tanpa kebenaran anda.

Sampel calitan permukaan mulut dan najis hanya akan dianalisa mengikut kajian/prosedur yang disebutkan dalam protokol dan tidak melepasi apa yang dinyatakan. Mana-mana sisa calitan mulut dan najis akan dihapuskan.

Kajian ini tidak akan mengakibatkan penyakit pada masa ini, dan maklumat yang dikumpulkan akan hanya diberitahu melalui permohonan rasmi.

MANFAAT YANG DIJANGKAKAN

(Manfaat kepada individu, masyarakat, dan universiti)

Dengan maklumat yang didapati, profil organisma usus akan digunakan sebagai rujukan untuk kajian klinikal seterusnya di Kelantan.

Data yang sama akan digunakan sebagai rujukan oleh pusat-pusat lain di Malaysia kerana tiada data yang diperolehi sebelum ini.

Maklumat kajian ini akan menjadi kayu ukur percubaan klinikal yang dibuat oleh pusat-pusat di Malaysia.

SOALAN

Sekiranya anda mempunyai apa-apa soalan tentang kajian ini atau hak-hak anda, sila hubungi:

Dr Nur Amalina Bt Muhammad No. Pendaftaran MMC 47693
Jabatan Perubatan Dalaman
Pusat Pengajian Sains Perubatan
USM Kampus Kesihatan, Kubang Kerian
Tel: 09-7676590/012-9049947

Sekiranya anda mempunyai soalan berkaitan Pengesahan Etika atau isu/masalah berkaitan dengan kajian ini, sila hubungi:

En Mohd Bazlan Hafidz Mukrim
Setiausaha Jawatankuasa Etika Kajian (Kemanusiaan)USM
Platform Kajian Sains Klinikal
Universiti Sains Malaysia
Kampus Kesihatan
No. tel.: 09-7672354/ 09-7672362
Email: bazlan@usm.my/jepem@usm.my

KERAHSIAAN

Maklumat perubatan anak anda akan dirahsiakan oleh doktor, kakitangan dan penyelidik dan tidak akan didedahkan kepada umum kecuali ia diperlukan dari segi undang-undang.

Data yang diperolehi daripada kajian ini tidak memperkenalkan anak anda secara individu dan akan diterbitkan untuk tujuan pengetahuan sahaja.
Rekod perubatan yang asal akan dipantau oleh penyelidik, Jawatankuasa Kajian Etika untuk kajian ini, dan pihak pengawalseliaan untuk tujuan pengesahan prosedur ujian percubaan klinikal dan / atau data.
Maklumat perubatan anak anda mungkin akan disimpan dan diproses dalam komputer.
Dengan menandatangani borang kebenaran ini, anda membenarkan tinjauan rekod, penyimpanan maklumat dan pertukaran data seperti yang digambarkan di atas.

TANDATANGAN

Untuk dilibatkan dalam kajian ini, anda atau wakil perundangan anda mesti menandatangani dan data mukasurat tandatangan

[LAMPIRAN S dan LAMPIRAN P]

LAMPIRAN S

Borang Maklumat ibubapa/penjaga dan Kebenaran (Mukasurat Tandatangan)

Tajuk kajian: Peranan pemakanan dan BMI pada profil organisma usus dalam kalangan kanak-kanak sekolah rendah di Kota Bharu, Kelantan

Nama penyelidik:Dr Nur Amalina Bt Muhammad (MPM 47693), Dr Wong Mung Seong(MPM45441) PM Lee Yeong Yeh,(MPM 36810), PM Norizan binti H A Majid(MPM 26234) PM Siti Asma binti Hassan(MPM34686), PM Chan Yean-Yean, PM Lee Yuan-Kun, & Dr Wong Pak Kai(MPM45546)

Untuk anak anda menjadi sebahagian daripada kajian ini, anda / wakil perundangan anda hendaklah menandatangani mukasurat ini. Dengan menandatangani mukasurat ini, saya mengesahkan perkara berikut:

- Saya sudah membaca kesemua maklumat di dalam Borang Maklumat dan Kebenaran Ibubapa termasuk mana-mana maklumat **berkaitan dengan risiko dalam kajian ini** dan saya sudah mengambil masa memikirkannya.
- Saya berpuashati dengan kesemua soalan yang dijawab.
- Saya secara sukarela bersetuju untuk anak saya menjadi sebahagian daripada kajian ini, akan mengikuti prosedur kajian, dan memberi maklumat yang diperlukan kepada doktor, jururawat, atau lain-lain staf, seperti yang diminta
- Saya mempunyai kebebasan untukanak saya berhenti daripada menjadi sebahagian daripada kajian ini pada bila-bila masa.
- Saya sudah menerima satu salinan Borang Maklumat dan Kebenaran Ibubapa untuk simpanan peribadi.

_____ _____

Nama Ibubapa/penjaga Nama pelajar

_____ _____

No.kad pengenalan ibubapa/penjaga No.kad pengenalan pelajar

_____ _____

Tandatangan Ibubapa atau wakil perundangan Tarikh (hh/bb/tt)(masa jika perlu)

_____ _____

Nama Individu
Yang mengadakan perbincangan kebenaran

_____ _____

Tandatangan Individu Tarikh (hh/bb/tt)
Yang Mengadakan Perbincangan Kebenaran

_____ _____

Nama dan Tandatangan Saksi Tarikh (hh/bb/tt)

Nota: i) Kesemua subjek/pesakit yang terlibat dalam kajian ini tidak dilindungi oleh insurans

LAMPIRAN P

Borang Kebenaran Penerbitan Bahan Pelajar
(Halaman Tandatangan)

Tajuk kajian: Peranan pemakanan dan BMI pada profil organisma usus dalam kalangan kanak-kanak sekolah rendah di Kota Bharu, Kelantan

Nama penyelidik:Dr Nur Amalina Bt Muhammad (MPM 47693), Dr Wong Mung Seong(MPM45441) PM Lee Yeong Yeh,(MPM 36810), PM Norizan binti H A Majid(MPM 26234) PM Siti Asma binti Hassan(MPM34686), PM Chan Yean-Yean, PM Lee Yuan-Kun, & Dr Wong Pak Kai(MPM45546)

Untuk anak anda menjadi sebahagian daripada penyelidikan ini, anda atau wakil perundangan anda hendaklah menandatangani halaman ini. Dengan menandatangani halaman ini, saya mengesahkan perkara berikut:

- Saya memahami bahawa nama anak saya tidak akan didedahkan dalam bahan-bahan yang diterbitkan dan terdapat usaha untuk memastikan kerahsiaan bahawa nama anak saya dirahsiakan walaupun kerahsiaan ini adalah tidak dijamin sepenuhnya kerana akibat-akibat yang tidak dapat dijangka.
- Saya sudah membaca bahan-bahan atau perihal umum tentang kandungan bahan dan meneliti kesemua foto-foto dan gambar-gambar termasuk kepunyaan anak saya yang boleh diterbitkan.
- Saya sudah ditawarkan peluang untuk membaca manuskrip dan melihat semua bahan yang melibatkan diri anak saya, tetapi telah mengecualikan hak saya untuk melihatnya.
- Semua bahan yang diterbitkan boleh dikongsi di kalangan pengamal perubatan, saintis dan wartawan di seluruh dunia.
- Kesemua bahan juga boleh gunakan dalam penerbitan tempatan, penerbitan buku-buku, dan diakses oleh ramai doktor tempatan dan antarabangsa seluruh dunia
- Saya dengan ini bersetuju dan membenarkan bahan-bahan ini untuk digunakan oleh penerbitan-penerbitan lain yang diperlukan oleh penerbit dengan syarat-syarat berikut:
    - ✓ Bahan-bahan ini tidak akan digunakan untuk tujuan periklanan atau bahan pembungkusan
    - ✓ Bahan-bahan ini tidak akan digunakan di luar konteks – i.e. : Contoh gambar tidak akan digunakan di dalam artikel yang tidak berkaitan dengan subjek di dalam gambar.

_____ _____

Nama Ibubapa/penjaga Nama Pelajar

_____ _____ _____

No. Kad pengenalan ibubapa/penjaga Tandatangan ibubapa Tarikh

_____ _____

Nama dan Tandatangan Individu
Yang mengadakan perbincangan kebenaran Tarikh (hh/bb/tt)

Nota: i) Kesemua subjek/pesakit yang terlibat dalam kajian ini tidak dilindungi oleh insurans.

Jawatankuasa Etika Penyelidikan Manusia USM (JEPeM)
Human Research Ethics Committee USM (HREC)

Universiti Sains Malaysia
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15th March 2016

014-3339638
Dr. Wong Mung Seong
Department of Medicine
School of Medical Sciences
Universiti Sains Malaysia
16150 Kubang Kerian, Kelantan.

JEPeM Code : USM/JEPeM/15110494
Protocol Title : Role of Diet and BMI on Basal GUT Microbiota Profile in Primary School Children in Kota Bharu.

Dear Dr.,

We wish to inform you that your study protocol has been reviewed and is hereby granted approval for implementation by the Jawatankuasa Etika Penyelidikan Manusia Universiti Sains Malaysia (JEPeM-USM). Your study has been assigned study protocol code **USM/JEPeM/15110494**, which should be used for all communication to the JEPeM-USM related to this study. This ethical clearance is valid from **March 2016** until **February 2017.**

The following researchers also involve in this study:

1. Dr. Nur Amalina Muhammad
2. Assoc. Prof. Dr. Lee Yeong Yeh
3. Assoc. Prof. Dr. Lee Yuan Kun
4. Assoc. Prof. Dr. Siti Asma Hassan
5. Assoc. Prof. Dr. Noorizan H A Majid
6. Assoc. Prof. Dr. Chan Yean Yean
7. Dr. Wong Pak Kai

The following documents have been approved for use in the study.

1. Research Proposal

In addition to the abovementioned documents, the following technical document was included in the review on which this approval was based:

1. Parent/Guardian Information Sheet and Consent Form (English version)
2. Parent/Guardian Information Sheet and Consent Form (Malay version)
3. Assent Form (Malay version)
4. Questionnaires – Soal Selidik Kekerapan Makanan bagi Profil Kajian Gut Microbiata

Attached document is the list of members of JEPeM-USM present during the full board meeting reviewing your protocol.

While the study is in progress, we request you to submit to us the following documents:

1. Application for renewal of ethical approval 60 days before the expiration date of this approval through submission of **JEPeM-USM FORM 3(B) 2014: Continuing Review Application Form.** Subsequently this need to be done yearly as long as the research goes on.
2. Any changes in the protocol, especially those that may adversely affect the safety of the participants during the conduct of the trial including changes in personnel, must be submitted or reported using **JEPeM-USM FORM 3(A) 2014: Study Protocol Amendment Submission Form.**

3. Revisions in the informed consent form using the **JEPeM-USM FORM 3(A) 2014: Study Protocol Amendment Submission Form.**
4. Reports of adverse events including from other study sites (national, international) using the **JEPeM-USM FORM 3(G) 2014: Adverse Events Report.**
5. Notice of early termination of the study and reasons for such using **JEPeM-USM FORM 3(E) 2014.**
6. Any event which may have ethical significance.
7. Any information which is needed by the JEPeM-USM to do ongoing review.
8. Notice of time of completion of the study using **JEPeM-USM FORM 3(C) 2014: Final Report Form.**

Please note that forms may be downloaded from the JEPeM-USM website: **www.jepem.kk.usm.my**

Jawatankuasa Etika Penyelidikan (Manusia), JEPeM-USM is in compliance with the Declaration of Helsinki, International Conference on Harmonization (ICH) Guidelines, Good Clinical Practice (GCP)

Standards, Council for International Organizations of Medical Sciences (CIOMS) Guidelines, World Health Organization (WHO) Standards and Operational Guidance for Ethics Review of Health-Related Research and Surveying and Evaluating Ethical Review Practices, EC/IRB Standard Operating Procedures (SOPs), and Local Regulations and Standards in Ethical Review.

Thank you.

"ENSURING A SUSTAINABLE TOMORROW"

Very truly yours,

PROF. DR. HANS AMIN VAN ROSTENBERGHE
Chairperson
Jawatankuasa Etika Penyelidikan (Manusia) JEPeM
Universiti Sains Malaysia

Jawatankuasa Etika Penyelidikan Manusia USM (JEPeM)
Human Research Ethics Committee USM (HREC)

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Date of meeting : 20 January 2016
Venue : Meeting Room, Centre for Research Initiatives, Clinical and Health Sciences, USM Kampus Kesihatan.
Time : 9.00 a.m – 3.00 p.m
Meeting No : 325

Members of Committee of the Jawatankuasa Etika Penyelidikan (Manusia), JEPeM Universiti Sains Malaysia who reviewed the protocol/documents are as follows:

Member (Title and Name)		Occupation (Designation)	Male/ Female (M/F)	Tick (✓) if present when above items, were reviewed
Chairperson : Professor Dr. Hans Amin Van Rostenberghe		Chairperson of Jawatankuasa Etika Penyelidikan (Manusia), JEPeM USM	M	✓ (Chairperson)
Secretary: Mr. Mohd Bazlan Hafidz Mukrim		Research Officer	M	✓
Members :				
1.	Professor Dr. Suzina Sheikh Abd Hamid	Lecturer, School of Medical Sciences	F	✓
2.	Associate Professor Dr. Mohtar Ibrahim	Lecturer, School of Medical Sciences	M	✓
3.	Associate Professor Dr. Lee Yeong Yeh	Lecturer, School of Medical Sciences	M	✓
4.	Associate Professor Dr. Nor Azwany Yaacob	Lecturer, School of Medical Sciences	F	✓
5.	Associate Professor Dr. Sarimah Abdullah	Lecturer, School of Medical Sciences	F	✓
6.	Associate Professor Oleksandr Krasilshchikov	Lecturer, School of Health Sciences	M	✓
7.	Associate Professor Siti Hawa Ali	Lecturer, School of Health Sciences	F	✓
8.	Dr. Soon Lean Keng	Lecturer, School of Health Sciences	F	✓
9.	Dr. Azlan Husin	Lecturer, School of Medical Sciences	M	✓
10.	Mr. Haji Ismail Hassan	Community Representative	M	✓
11.	Mr. Harry Mulder	Community Representative	M	✓
12.	Mrs. Zawiah Abu Bakar	Community Representative	F	✓

Jawatankuasa Etika Penyelidikan (Manusia), JEPeM-USM is in compliance with the Declaration of Helsinki, International Conference on Harmonization (ICH) Guidelines, Good Clinical Practice (GCP) Standards, Council for International Organizations of Medical Sciences (CIOMS) Guidelines, World Health Organization (WHO) Standards and Operational Guidance for Ethics Review of Health-Related Research and Surveying and Evaluating Ethical Review Practices, EC/IRB Standard Operating Procedures (SOPs), and Local Regulations and Standards in Ethical Review.

PROFESSOR DR. HANS AMIN VAN ROSTENBERGHE
Chairperson
Jawatankuasa Etika Penyelidikan (Manusia), JEPeM
Universiti Sains Malaysia

Soal Selidik Kekerapan Makanan Bagi Profil Kajian Organisma Usus.

Untuk kegunaan rasmi sahaja

Nama :

Umur :

Bangsa ;

Jantina :

Bahagian A

Sila bulatkan SATU jawapan paling tepat

1. Adakah anda mengambil ubat-ubatan?

1 Tidak
2 Ya
Jika ya, sila nyatakan (contoh, antibiotic)

2. Adakah diet anda berubah beberapa tahun kebelakangan ini?

1 Tidak
2 Ya
Jika ya, bagaimanakah ianya bertukar? Sila nyatakan

3. Apakah kekerapan anda makan makanan dengan kari, contohnya kari sayur, kari ikan atau daging?
_____ setiap minggu/bulan*
Lain-lain: _____

4. Apabila anda makan makanan dengan lemak yang dapat dilihat, berapa banyakkah lemak yang dilihat yang anda singkirkan?

1 Semua
2 Sedikit
3 Tiada
4 Saya tidak makan daging langsung

5 Apabila anda memakan ayam (contohnya ayam, itik, ayam belanda, merpati, dll), berapa banyakkah kulitnya yang dibuang?

1 Semua
2 Sedikit
3 Tiada
4 Saya tidak makan ayam langsung

6. Apakah jenis minyak/lelemak yang selalu digunakan keluarga anda untuk memasak (goreng dalam kuali, menggoreng)?Pilih satu daripada pilihan daripada senarai yang diberi.

0. Sayur yang dikisar (minyak masak)
1. Minyak politaktepu (jagung, soya, bunga matahari,safflower, grapeseed,flaxseed)
2. Minyak politaktepu (zaitun, kekacang, canola, dedak padi, bijan, sawi)
3. Lemak tepu (lard, ghee, tallow, marjerin keras,mentega, minyak kelapa, minyak sawit)
4. Tidak menggoreng dalam kuali atau menggoreng

7. Apakah jenis minyak/lelemak yang sering keluarga anda gunakan untuk memasak (menggoreng)? Pilih satu daripada pilihan dalam senarai.

0. Minyak sayuran yang dikisar (minyak masak)
1. Minyak politaktepu (jagung, soya, bunga matahari, safflower, grapeseed, flaxseed)
2. Minyak monotaktepu (zaitun, kacang, canola, dedak padi, bijan, sawi)
3. Lemak tepu (lard, ghee, tallow, marjerin keras, mentega, shortening, minyak kelapa,minyak sawit)
4. Tidak menggoreng

8. Apakah jenis minyak/lelemak yang sering keluarga anda gunakan untuk sos salad?

0. Minyak sayuran yang dikisar (minyak masak)
1. Minyak politaktepu (jagung, soya, bunga matahari, safflower, grapeseed, flaxseed)
2. Minyak monotaktepu (zaitun, kacang, canola, dedak padi, bijan, sawi)
3. Lemak tepu (lard, ghee, tallow, marjerin keras, mentega, shortening, minyak kelapa,minyak sawit)
4. Tidak menggunakan sos salad

9. Apakah jenis susu yang seeing anda gunakan dengan kopi atau teh? Pilih satu daripada pilihan dalam senarai.

0. Krimer
1. Susu isian manis
2. Susu sejat
3. Susu penuh krim(susu segar)/tepung
4. Susu rendah lemak (susu segar rendah lemak)/tepung
5. Susu skim/tepung
6. Tiada susu
7. Tidak minum kopi atau teh

10. Apakah jenis susu yang sering anda gunakan dengan minuman malt (contohnya Horlicks, Milo)? Pilih satu pilihan daripada senarai.

0. Krimer
1. Susu isian manis
2. Susu sejat
3. Susu penuh krim(susu segar)/tepung
4. Susu rendah lemak (susu segar rendah lemak)/tepung
5. Susu skim/tepung
6. Tiada susu
7. Tidak minum minuman malt

BAHAGIAN B

ARAHAN

Sila ingat semula dan rekod makanan yang telah anda makan **setahun lepas**. Sila catat sekerap mana anda makan sesetengah makanan berserta dengan jumlahnya. Anda hanya perlu memasukkan makanan yang telah dimakan dan jangan memasukkan makanan lebihan atau tumpah. Bagi sesetengah makanan, sila rujuk kepada gambar untuk membantu anda membuat anggaran.

Untuk membantu anda memulakannya, di sini disenaraikan contoh yang dimaksudkan. Sekiranya anda boleh mengambil beberapa minit untuk meneliti contoh-contoh ini, ianya mungkin dapat membantu anda.

CONTOH:Sekerap manakah anda makan roti putih?

Sekiranya anda sudah makan nasi putih sehari, anda patut merekod di bawah kolum 1. Sila nyatakan juga jumlah purata yang diambil setiap kali. Rujuk gambar makanan sekiranya ada. **Untuk peringatan, anda hanya diminta mengisi hanya satu kolum, sama ada secara bulanan harian atau tidak pernah**.

Jenis makanan	Jumlah kekerapan makan				
Berapa kerap anda makan makanan berikut	Tidak pernah	Sebulan	Seminggu	Sehari	Jumlah purata per hidangan
Nasi					
Nasi putih (gambar A1-A3)				**1**	Gambar =**A2** atau bil sudu kecil :

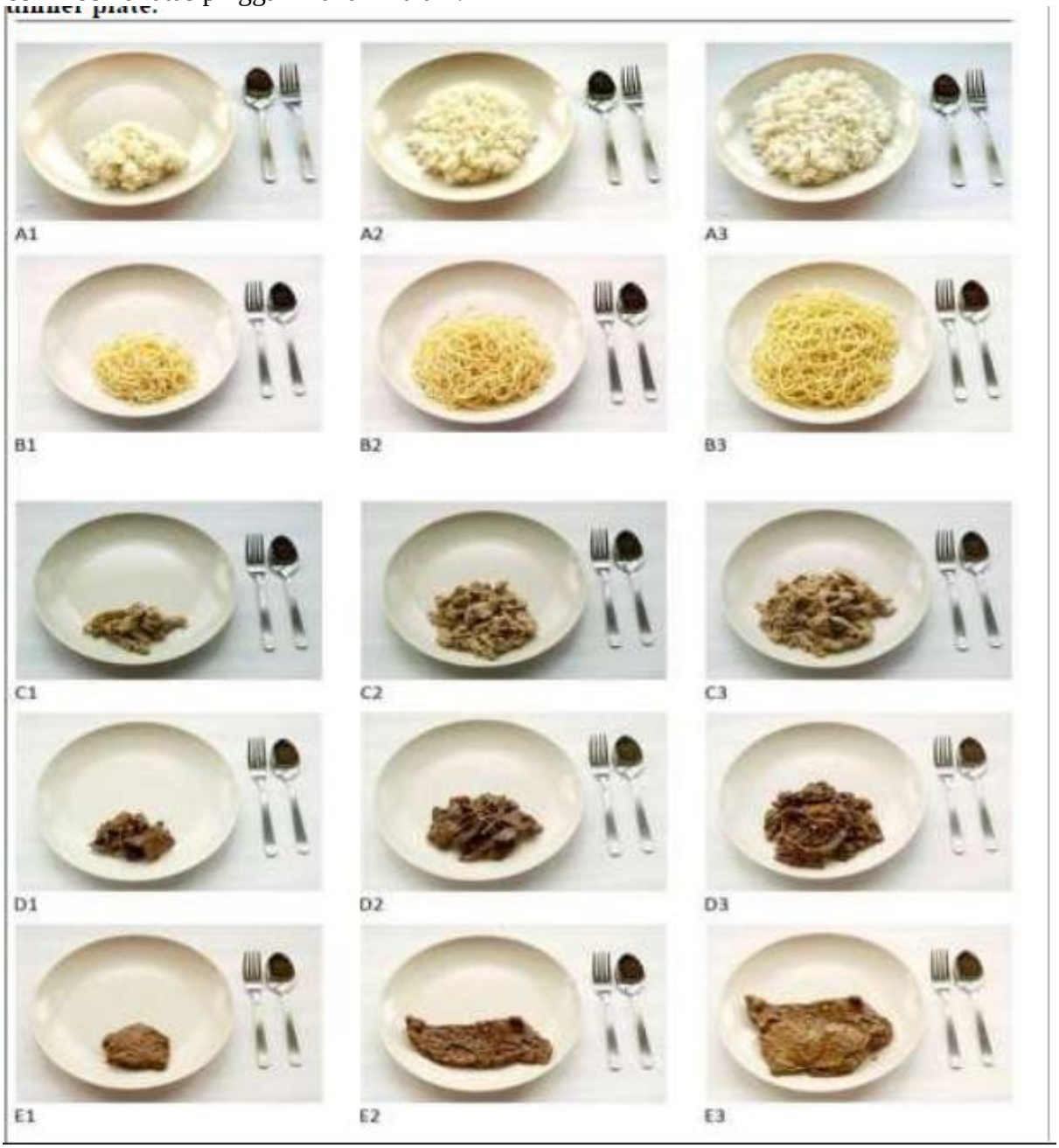
Sekiranya anda tidak makan nasi putih, tandakan bahagian **tidak pernah**. Jangan tinggal tempat kosong.

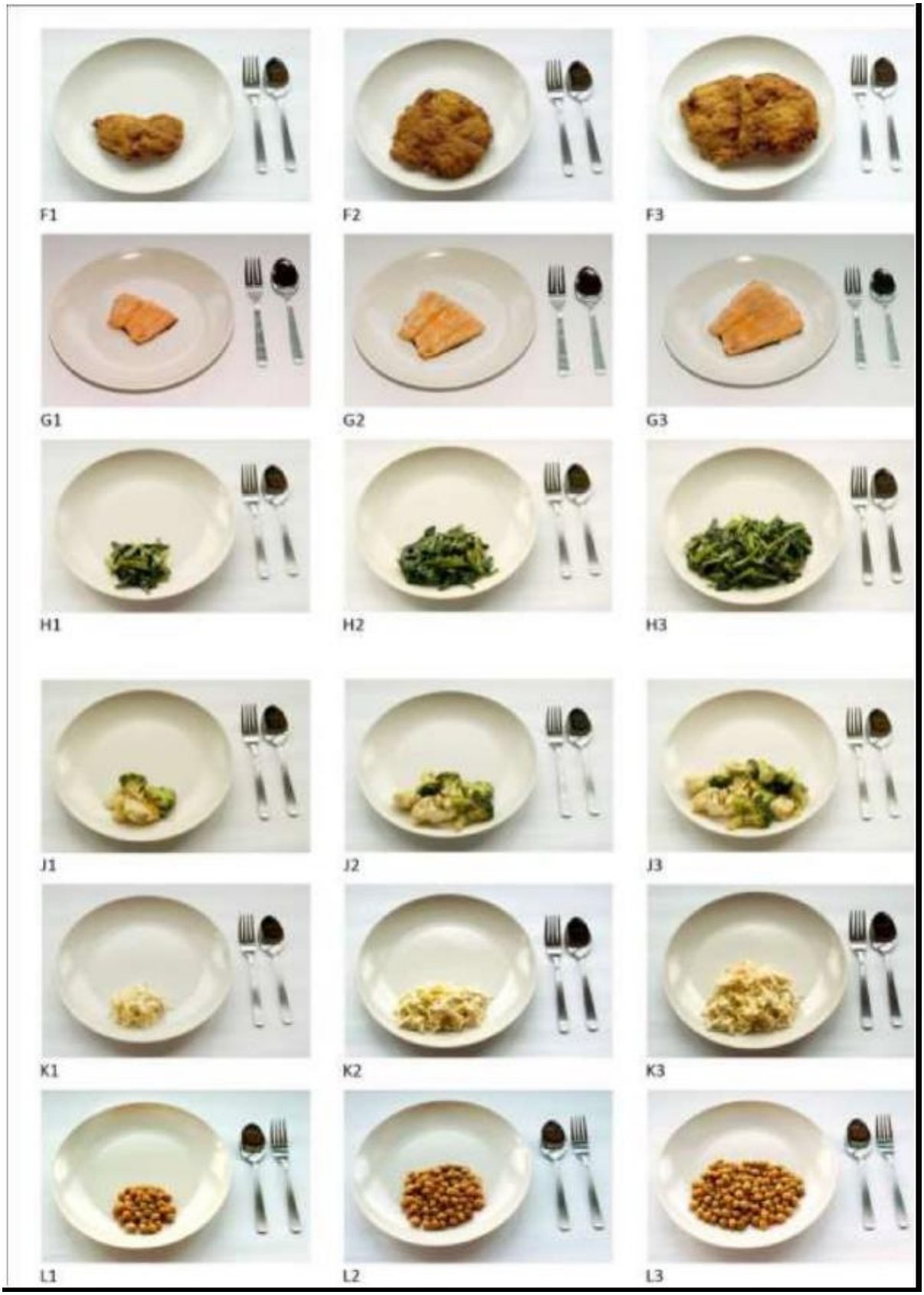
Jenis makanan	Jumlah kekerapan makan				
Berapa kerap anda makan Makanan berikut	Tidak pernah	Sebulan	Seminggu	Sehari	Jumlah purata per hidangan
Nasi					
1 Nasi putih [gambar A1-A3]					Gambar =_ atau bil sudu kecil :

Gambar i.e A2,B2,C2,D2 sehingga ke R2 menunjukkan hidangan normal bagi makanan yang disediakan di gerai .Berdasarkan pengetahuan terbaik anda, nyatakan gambar yang paling sesuai dengan makanan yang anda makan. Walaubagaimanapun sekiranya tiada gambar untuk sesetengah barangan makanan, sila anggarkan dengan bilangan sudu kecil.

GAMBAR MAKANAN UNTUK MENGANGGARKAN SAIZ HIDANGAN

Gunakan gambar-gambar di bawah untuk menyatakan makanan yang telah anda makan. Gambar-gambar ini boleh digunakan untuk makanan yang tidak dipaparkan. Sila nyatakan nombor gambar dan saiz terdekat dengan hidangan yang anda makan untuk membantu. Hidangan makan malam menggunakan pinggan 10 inci. Kek digambarkan dengan pinggan lebih kecil di atas pinggan makan malam.




F1
F2
F3
G1
G2
G3
H1
H2
H3
J1
J2
J3
K1
K2
K3
L1
L2
L3

M1

M2

M3

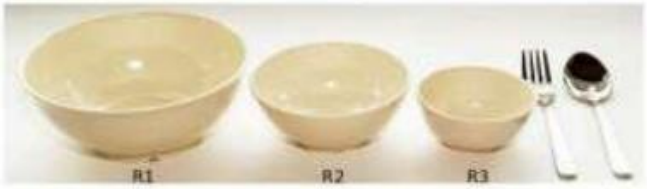
N1

N2

Q1

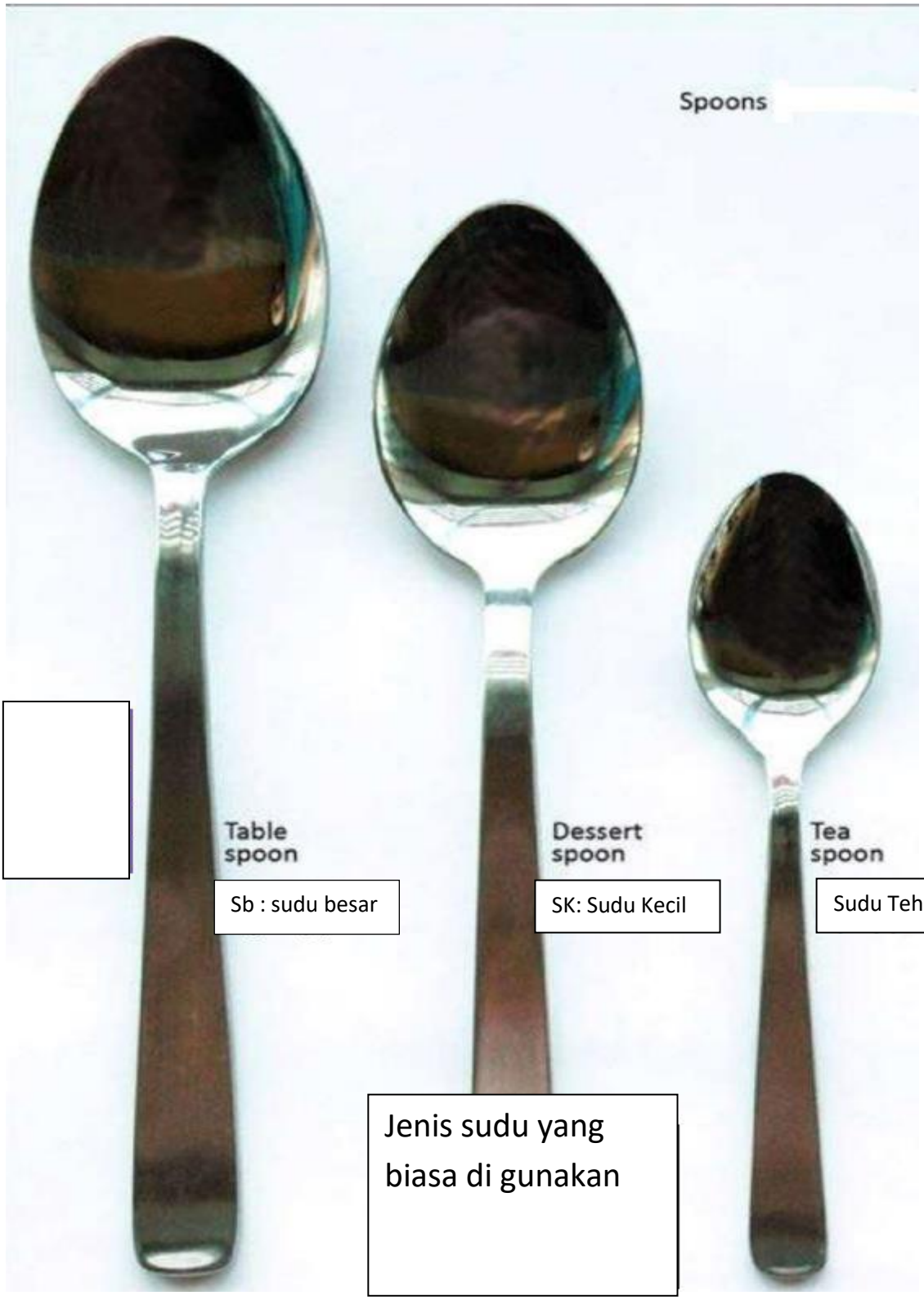
Q2

Q3



Bowl Code	Diameter	Volume
R1	17.5cm	750ml
R2	12.5cm	300ml
R3	9cm	250ml

S1 **S2** **S3** **S4** **S5** **S6** **S7** **S8** **S9**



Sb : sudu besar

SK: Sudu Kecil

Sudu Teh

Jenis sudu yang biasa di gunakan

<u>Pastikan anda memahami arahan sebelum memulakan soal selidik kekerapan makanan</u>.

Bahan Makanan		**Kekerapan makan**				
Berapa kerapkah anda mengambil makanan berikut?		Tidak pernah	Sebulan	Seminggu	Sehari	Jumlah purata setiap hidangan
Roti						
1	Roti putih					Jumlah kepingan:
2	Roti gandum penuh					Jumlah kepingan:
3	Roti dengan buah-buahan dan kekacang					Jumlah kepingan:
Sapuan roti yang dipilih						
4	Mentega					Jumlah sb(sudu besar);
5	Marjerin					Jumlah sb:
6	Mentega kacang					Jumlah sb:
7	Jem/Madu					Jumlah sb:
8	Kaya					Jumlah sb:
Lain-lain jenis roti						
9	Roti parata/murtabak					Jumlah kepingan:
10	Chapati/tosay/naan					Jumlah kepingan:
11	Roti ban dengan kelapa/isi daging					Jumlah ban:
12	Roti ban dengan isi manis (strawberi, kaya, etc)					Jumlah ban:
Bijirin						
13	Tiada perisa/bijirin sarapan berperisa					Jumlah sk(sudu kecil);
14	Oat/oatmeal (mentah)					Jumlah sk
Nasi dan bubur						
15	Nasi putih masak kosong [gambar A1-A3]					Gambar=___ atau jumlah sk:
16	Bubur kosong (dimasak dengan nasi putih sahaja)					Jumlah sk:
17	Nasi goreng/Nasi claypot [gambar A1-A3 atau b1-b3]					Gambar=___ atau jumlah sk

Bahan Makanan		**Kekerapan makan**				
Berapa kerapkah anda mengambil makanan berikut?		Tidak pernah	Sebulan	Seminggu	Sehari	Jumlah purata setiap hidangan
Nasi/bubur						
18	Ayam/nasi itik [gambar A1-A3]					Gambar=___ atau jumlah sk (sudu kecil) :
19	Nasi biryani/nasi lemak [gambar A1-A3]					Gambar=___ atau jumlah sk
20	Bubur berperisa (contohnya ayam, khinzir, itik, ikan)					Gambar=___ atau jumlah sk
Mee sup						
21	Mee sup nasi (contohnya bihun, kway teow, hor fun) dengan daging lembu/ayam/bebola ikan [gambar R1-R3]					Gambar=___ atau jumlah sk
22	Mi sup gandum (contohnya mi, udon, ramen) dengan daging lembu/ayam/bebola ikan [gambar R1-R3]					Gambar=___ atau jumlah sk
Mi Kering						
23	Mi beras kering (contohnya bihun, kway teow, hor fun) dengan daging lembu/ayam/bebola ikan [gambar R1-R3]					Gambar=___ atau jumlah sk
24	Mi gandum kering contohnya mi, udon, ramen) dengan daging lembu/ayam/bebola ikan [gambar R1-R3]					Gambar=___ atau jumlah sk
Mi Goreng						
25	Bihun goreng (mi beras) [gambar B1-B3]					Gambar=___ atau jumlah sk
26	Mi goreng gandum contohnya mi hokkien, mi goreng [gambar B1-B3]					Gambar=___ atau jumlah sk
Mi berkuah						
27	Mi beras berkuah (contohnya mi siam, hor fun, laksa) [gambar B1-B3]					Gambar=___ atau jumlah sk
28	Mi gandum berkuah (contohnya mi rebus) [gambar B1-B3]					Gambar=___ atau jumlah sk

Bahan Makanan		**Kekerapan makan**				
Berapa kerapkah anda mengambil makanan berikut?		Tidak pernah	Sebulan	Seminggu	Sehari	Jumlah purata setiap hidangan
Lain-lain jenis mi						
29	Mi segera [gambar R1-R3]					Gambar=___ atau jumlah sk (sudu kecil)
30	Rebus/spaghetti/pasta (kosong) [gambar B1-B3]					Gambar=___ atau jumlah sk
31	Mi rebus/spaghetti/pasta dengan sos krim putih [gambar B1-B3]					Gambar=___ atau jumlah sk
Sup						
32	Krim sup [gambar R1-R3]					Gambar=___ atau jumlah sk
33	Sup putih/sup [gambar R1-R3]					Gambar=___ atau jumlah sk
Rempah/Herba						
34a	Serbuk cili					TB(Tidak berkenaan)
34b	Serbuk kari					TB
34c	Halia					TB
34d	Parsli					TB
34e	Kunyit					TB
34f	Ketumbar					TB
34g	Rosemary					TB
34h	Rumpai Dill					TB
34i	Daun pudina					TB
34j	Lain-lain					TB

Bahan Makanan		**Kekerapan makan**				
Berapa kerapkah anda mengambil makanan berikut?		Tidak pernah	Sebulan	Seminggu	Sehari	Jumlah purata setiap hidangan
Perasa/Sos						
35a	Bawang					TB (Tidak berkenaan)
35b	Lada					TB
35c	Sos cili					TB
35d	Lain-lain					TB
Sayur-sayuran dan Tauhu						
36	Goreng/kosong					Gambar=___ atau jumlah sk (Sudu kecil)
37	Mentah/stim/dalam sup					Gambar=___ atau jumlah sk
Sayuran berdaun hijau gelap						
38	Goreng/kosong					Gambar=___ atau jumlah sk
39	Mentah/stim/dalam sup					Gambar=___ atau jumlah sk
Tomato,lobak, lada benggala merah/kuning						
40	Goreng/kosong					Gambar=___ atau jumlah sk
41	Mentah/stim/dalam sup					Gambar=___ atau jumlah sk
Kekacang, contohnya kacang dan kacang pis						
42	Goreng/kosong					Gambar=___ atau jumlah sk
43	Kekacang kering (contohnya dhal, kacang kering)dalam kuah [gambar L1-L3]					Gambar=___ atau jumlah sk
44	Mentah/stim/rebus					Gambar=___ atau jumlah sk

Bahan Makanan		**Kekerapan makan**				
Berapa kerapkah anda mengambil makanan berikut?		Tidak pernah	Sebulan	Seminggu	Sehari	Jumlah purata setiap hidangan
Sayur campur						
45	Goreng/kosong					Gambar=___ atau jumlah sk
46	Goreng dalam minyak (contohnya tempura) [gambar H1-H3]					Gambar=___ atau jumlah sk
47	Kari/Lemak [gambar H1-H3]					
48	Mentah/stim/dalam sup/Rojak [gambar H1-H3]					Gambar=___ atau jumlah sk
Tauhu dan taukua						
49	Goreng					Jumlah sk (sudu kecil);
50	Stim/dalam sup					Jumlah sk
Akar/Batang (cth: ubi kentang, keledek, jagung)						
51	Goreng					Jumlah sk
52	Sup dengan daging stu/stok					Jumlah sk
Sayur jeruk						
53	Contohnya Chye sim, zaitun [gambar H1-H3]					Gambar=___ atau jumlah sk
Sayur tapai						
54	Contohnya Kimchi, Tampeh, Natto [gambar H1-H3]					Gambar=___ atau jumlah sk
Ayam itik tanpa kulit [gambar C1-C3] contohnya Ayam/Itik						
55	Goreng/Stu/panggang/bakar					Gambar=___ atau jumlah sk
56	Goreng pan					Gambar=___ atau jumlah sk
57	Stim					Gambar=___ atau jumlah sk

Bahan Makanan		**Kekerapan makan**				
Berapa kerapkah anda mengambil makanan berikut?		Tidak pernah	Sebulan	Seminggu	Sehari	Jumlah purata setiap hidangan
Daging, contohnya : Daging Lembu/ khinzir/ Kambing						
58	kacau goreng, rebus, tumis, panggang dan bakar					Gambar=___ atau jumlah sk
59	Goreng pan dan goreng minyak penuh					Gambar=___ atau jumlah sk
60	Stim/dalam sup					Gambar=___ atau jumlah sk
Daging terawet/BBQ						
61	Sosej, sila nyatakan jenis (contohnya, ayam,daging lembu atau daging khinzir) ; _____					Jumlah:
62	Daging paha salai/daging khinzir, bakon/dalam tin					Jumlah keping:
63	Hati atau lain-lain organ dalaman					Jumlah sk:
64	Daging jerky (contohnya daging BBQ, Bak Kua, stik daging)					Jumlah sk:
Ikan/Makanan laut (Ikan tidak berminyak contohnya)						
65	Goreng/Bakar					Gambar=___ atau jumlah sk
66	Goreng pan/digoreng/digoreng dengan adunan					Gambar=___ atau jumlah sk
67	Stim/Asam pedas					Gambar=___ atau jumlah sk
68	Kari Kelapa [gambar G1-G3]					Gambar=___ atau jumlah sk
69	Bertin (contohnya tuna) [gambar G1-G3]					Gambar=___ atau jumlah sk
Lain-lain jenis makanan laut (contohnya udang)						
70	Goreng/Bakar					Jumlah sk:
71	Goreng pan/digoreng/Digoreng dengan adunan					Jumlah sk:
72	Stim/Asam pedas					Jumlah sk:
73	Kari kelapa					Jumlah sk:
74	Makanan laut mentah (contohnya salmon, sotong kurita)					Jumlah sk:

Bahan Makanan		**Kekerapan makan**				
Berapa kerapkah anda mengambil makanan berikut?		Tidak pernah	Sebulan	Seminggu	Sehari	Jumlah purata setiap hidangan
Susu dan produk tenusu						
90	Susu prnuh krim (segar,UHT,tepung) [gambar S1-S9]					Gambar=____atau jumlah hidangan:
91	Susu rendah lemak(segar,UHT,tepung) [gambar S1-S9]					Gambar=____atau jumlah hidangan:
92	Susu skim (segar,UHT,tepung) [gambar S1-S9]					Gambar=____atau jumlah hidangan:
93	Keju rendah lemak					Jumlah potongan:
94	Yogurt biasa					Jumlah sk:___ atau berat dalam gram:
95	Yogurt rendah lemak (termasuk yogurt beku)					Jumlah sk:___ atau berat dalam gram:
96	Keju					Jumlah potongan:
97	Sapuan keju					Jumlah sk:__ atau segitiga
98	Lain-lain [gambar S1-S9] (contohnya yakult, vitagen, lassi, minuman yogurt,shake susu buah-buahan) sila nyatakan: _____					Gambar=____atau jumlah hidangan:
Produk soya						
99	Susu soya (segar/paket/tin) [gambar S1-S9]					Gambar=____atau jumlah hidangan:
100	Tofu soya (tau huay)					Jumlah sk:
Pencuci Mulut **Pencuci mulut bersup**						
101	Dengan santan/krim (contohnya pulau hitam, bubor cha cha) [gambar R1-R3]					Gambar=____atau jumlah hidangan:
102	Tanpa santan (contohnya cheng teng, sup kacang hijau tau suan) [gambar R1-R3]					Gambar=____atau jumlah hidangan:

Bahan Makanan		**Kekerapan makan**				
Berapa kerapkah anda mengambil makanan berikut?		Tidak pernah	Sebulan	Seminggu	Sehari	Jumlah purata setiap hidangan
Kuih-muih-stim						
103	Dengan kelapa/santan/krim kelapa (contohnya kuih sarlat, kuih dadar, putu mayam, idli)					Jumlah keping:
104	Tanpa santan (kuih tutu, soon kway)					Jumlah keping:
105	Snek bergoreng (contohnya goreng pisang you tiao, rojak India) Sila nyatakan: _____					Jumlah keping:
106	Dim Sum-stim (contohnya chee cheong fun, ladu, ladu beras)					Jumlah keping:
107	Dim sum-goreng/bergoreng (contohnya kek lobak merah goreng, wanton, puff char siew)					Jumlah keping:
108	Snek India manis (contohnya burfi, halwa)					Jumlah keping:
Biskut, pastry dan kek						
109	Biskut biasa					Jumlah keping:
110	Biskut berkrim/shortbread					Jumlah keping:
111	Puff/pastri kelopak (croissant, karipap bakar, dll)					Jumlah keping:
112	Kek mentega biasa/kek buah-buahan [gambar Q1-Q2]					Gambar=____atau jumlah potongan:
113	Kek span [gambar Q1-Q2]					Gambar=____atau jumlah potongan:
114	Kek berkrim [gambar Q1-Q2]					Gambar=____atau jumlah potongan:
Makanan ringan dan snek						
115	Snek goreng masin (garing/keropok udang, keropok, biskut masin dll)					Jumlah keping:

Bahan Makanan		**Kekerapan makan**				
Berapa kerapkah anda mengambil makanan berikut?		Tidak pernah	Sebulan	Seminggu	Sehari	Jumlah purata setiap hidangan
116	Ais krim					Jumlah sk:
117	Coklat					Jumlah segiempat:__atau jumlah saiz snek:
Minuman manis						
118	Minuman manis (contohnya minuman ringan, minuman dalam paket, dalam tin atau dalam botol) [gambar S1-S9]					Gambar=____atau jumlah hidangan:
Lain-lain jenis minuman						
119	Kopi tanpa gula [gambar S1-S9]					Gambar=____atau jumlah hidangan:
120	Kopi bergula [gambar S1-S9]					Gambar=____atau jumlah hidangan:
121	Teh tanpa gula [gambar S1-S9]					Gambar=____atau jumlah hidangan:
122	Teh bergula [gambar S1-S9]					Gambar=____atau jumlah hidangan:
123	Teh hijau (teh tidak ditapai) [gambar S1-S9]					Gambar=____atau jumlah hidangan:
124	Teh hitam (teh tapai sepenuhnya [gambar S1-S9]					Gambar=____atau jumlah hidangan:
125	Teh Oolong (teh tapai separuh) [gambar S1-S9]					Gambar=____atau jumlah hidangan:
126	Minuman malt (contohnya coklat panas, Horlicks, Milo, Ovaltine) [gambar S1-S9]					Gambar=____atau jumlah hidangan:
Minuman Beralkohol						
127	Bir/Stout					Jumlah hidangan:
128	Wain					Jumlah hidangan:
129	Minuman keras					Jumlah hidangan:

Makanan		Berapa Kali dimakan				Kuantiti setiap kali dimakan (contoh 2 tablet)	Sudah berapa lama anda mengambil makanan tambahan ini?	Nyatakan jenama jika diketahui
Berapa kerap anda mengambil makanan tambahan ini?		Tidak Pernah	Sebulan	Seminggu	Sehari			
130a	Multivitamin dan mineral (contohnya centrum)	☐						
130b	Kalsium sahaja	☐						
130c	Vitamin C sahaja	☐						
130d	Vitamin D sahaja	☐						
130e	Kalsium dengan Vitamin D	☐						
130f	Asid lemak Omega-3 (minyak ikan)	☐						
130g	Lain-lain (sila nyatakan. Contohnya probiotik atau probiotik): _____	☐						

CHAPTER 5

APPENDICES

5.1 Elaboration of the methodology

1. Preparation of Fecal Sample

1.1. Fecal Sampling (by the donor)

Collect the fecal sample according to the following procedure.

Materials

- Fresh feces
- Fecal collection tube
- Glass beads (ø 2.5 mm)
- RNA*later*®
- Trail paper

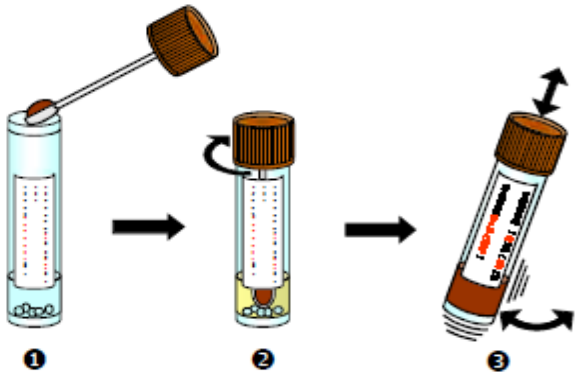
➢ Fecal Collection Tube..... Take 2 ml RNA*later*® and 5 ~ 10 glass beads (ø 2.5 mm) in a tube, and measure the weight prior to fecal sampling.

Procedure

1) Float a trail paper on water surface in a lavatory bowl. Refer to Picture 1.
2) Using the spatula attached to the tube cap, take one spatula portion (0.3 ~ 0.5 g) of the feces defecated in the trail paper. Avoid taking feces dipped with urine or water (Fig. 1❶).
3) Put the spatula into the fecal collection tube, and cap the tube tightly (Fig. 1❷).
4) Shake the tube vigorously for 10 seconds to suspend the feces in RNA*later*® (Fig. 1❸).
5) Store it at 4 °C within 3 weeks. This series of steps have to be performed as soon as possible after the defecation.

Picture 1

Fig. 1. Fecal sampling



5.2 DNA extraction

The total bacterial DNA extraction was performed using QIAamp® Fast DNA stool mini kit (https://www.qiagen.com) with minor modification. Purification of DNA from stool samples was automated on the QIAcube (Figure 2).In brief, 180–220 mg weighed stool into a 2 ml microcentrifuge tube and tube was placed on ice. 1 ml InhibitEX Buffer was added to each stool sample and vortex continuously for 1 min or until the stool sample was thoroughly homogenized. At that point, the suspension was heated for 5 min at 70°C, vortex for 15 s. The sample was centrifuged at full speed for 1 min to pellet stool particles, and 15 µl proteinase K was pipet into a new 1.5 ml microcentrifuge tube. About 200 µl supernatant was pipetted from step 4 into the 1.5 ml microcentrifuge tube containing proteinase K and add 200 µl Buffer AL and vortex for 15 s then incubate at 70°C for 10 min. 200 µl of ethanol (96–100%) was added to the lysate, and mix by vortexing. 600 µl lysate was applied from step 9 to the QIAamp spin column and was centrifuged at full speed for 1 min. QIAamp spin column was placed into a new 2 ml collection tube, and tube containing the filtrate were discard. 500 µl Buffer AW1 was added after carefully open the QIAamp spin column and then centrifuged at full speed for 1 min. The QIAamp spin column was placed into a new 2 ml collection tube, and the filtrate was discarded. By open the QIAamp spin column, 500 µl Buffer AW2 was added and centrifuged at full speed for 3 min. The collection tube containing the filtrate was discarded. QIAamp spin column was placed in a new 2 ml collection tube and the old collection tube with the filtrate was discared, then centrifuged at full speed for 3 min. the QIAamp spin column was transferred into a new, labeled 1.5 ml microcentrifuge tube and 200 µl Buffer ATE was pipetted directly onto the QIAamp membrane. Finally, it was incubated for 1 min at room temperature, and centrifuged at full speed for 1 min to elute DNA.

Figure 2; Automated DNA purification. DNA purification using the QIAamp Fast DNA Stool Mini Kit can be automated on QIAcube

5.3 Additional literature review

The human gut microbiota has gained increasing interest for its equivocal impact on human health, such as its comprehensive physiological and pathological function. (1-4). Modification in the proportions of gut microbes in the concentration of the compounds produced and released by them in the intestines have been suggested to play a role in the development of pathological condition including inflammatory bowel disease (IBD), colon cancer, obesity and dibetes mellitus.(5-7). The adult healthy gut microbiota is dominated with Firmicutes (gram positive) and Bacteroidetes (gram negative) phyla (8). Most common bacteria in adult human microbiota belong to Firmicutes and Bacteroidetes phylum is the second most common in human gut, with a predominance of the *Bacteroides* and *Prevotella* genera (8,9). Studies have shown that prevotella species is associated with gut inflammation, mainly mediated by proinflammatory Th17 cytokines, induce IL-8 and IL 6 secretion by epithelial cell, favoring Th 17 reponses and neutrophil recruitment.(10). Thus inflammation of gut mucosa mediated by prevotella spp, promotes systemic dissemination of inflammatory mediators, increased intestinal permeability and translocation of bacterial products, which amplified and promoted systemic inflammation.(10)

5.4 Additional references

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5.5 Raw data on SPSS softcopy