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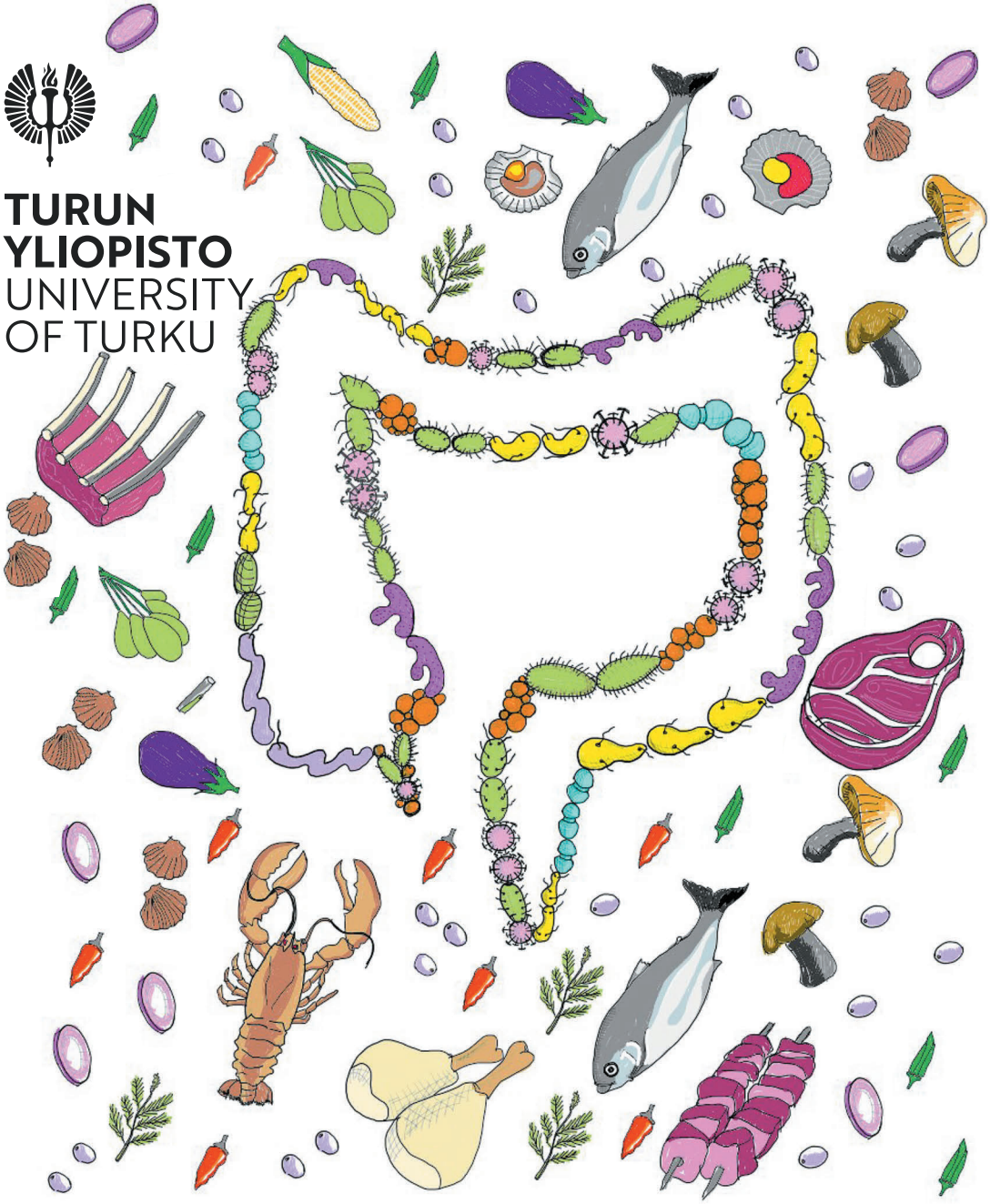

HUMAN MICROBIOTA: DETERMINING DIETARY FACTORS ON THE JOURNEY FROM INFANTS TO ADULTS

Wei Wei Thwe Khine

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ABSTRACT

The human microbiota profile changes with age from birth. The early colonisers of the infants' guts are vital for their current health and also that in later ages of their lives. Determining factors affecting gut microbiota composition across ages are also critical. The thesis aims to understand gut microbiota composition from infants to adults and access to diet as its major modulating factor in healthy children and adults. Faecal microbiota was assessed using 16s rRNA amplicon sequencing in the mother-child pairs (less than one month-48 months in Study I), children (7-12 years in Study II), and adults from two dietary intervention studies (18-40 years in Study III and IV). The first study in this thesis revealed that transmission of the unique gut microbiota, *Prevotella*, from Indonesian mothers to their infants was successfully established but developed slowly after the weaning period when the infants' gut microbiota, *Bifidobacterium* profile, changed to the adult-type, *Prevotella*. The weaning period (6-12 months old) is a remarkable period of alteration of gut microbiota, the immune responses (cytokines), and bile acids metabolism. After this, the diet is the primary factor influencing the gut microbiota of children. The second study disclosed that geographical location varies the gut microbiotas of Chinese and Malay children from China and two Malaysian cities into two clusters (*Bacteroides* and *Prevotella*). The location differences uncovered that the diet is the key influencing factor but not ethnicity. The third study showed that the gut microbiotas of Singaporean young adults could be divided into three clusters (*Bacteroides*, *Prevotella*, and *Blautia*) based on their dietary habits. The *Blautia* cluster (having randomly rotating Western/Southeast Asian foods) benefited from downregulated immune-regulation without changing its enterotype after the consumption of *Lactobacillus*-administered milk drink. The *Bacteroides* cluster resisted changing its enterotype and had a very slight response regarding the alteration of cytokine. The *Prevotella* cluster decreased its enterotype with upregulated (anti-inflammatory cells) and balanced immunoregulation. The final study found that the abundance of *Bacteroides* in the gut of Singaporean adults was significantly reduced following a polyphenol-rich meal containing mixed spices. Notably, the abundance of *Bifidobacterium* was enhanced after a meal with a high-dose of spices.

Understanding gut microbiota development in infants, children, and adults and modifying its composition with beneficial microbes (probiotics) and polyphenols may promote the design of new dietary regimens and foods. These may have beneficial effects on health.

KEYWORDS: *Bifidobacterium*, *Prevotella*, Gut microbiota, Healthy dietary habit

TURUN YLIOPISTO
Lääketieteellinen tiedekunta, Kliininen laitos, Lastentautioppi
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TIIVISTELMÄ

Ihmisen mikrobistoprofiili muokkautuu syntymässä ja vaiheittain sen jälkeen. Lapsen suoliston varhainen kolonisaatio on merkittävä myöhemmän terveyden kannalta. Suoliston mikrobiston koostumukseen vaikuttavat mm. lapsen ikä, syntymätapa, ympäristötekijät ja ravinto. Tämän tutkimuksen tavoitteena oli karakterisoida suoliston mikrobiston koostumus, sen muutokset ja ravintotekijöiden vaikutus eri ikäkausina. Mikrobistomääritykset tehtiin 16s rRNA amplicon sekvensointia käyttäen. Tutkittavia olivat osatutkimuksessa I äiti-lapsi parit (lapset 1-48 kk), osatutkimuksessa II lapset (7-12 v) ja osatutkimuksissa III ja IV aikuiset (18-40 v). Ensimmäisessä tutkimuksissa todettiin, että Indonesialaisila äideillä vallitseva mikrobisuku on *Prevotella*, joka siirtyy herkästi lapselle imetyksen aikana. Vieroituksen jälkeen siirtymä aleni nopeasti. Samanaikaisesti imeväisikäisten lasten bifidobakteeri-valtainen mikrobisto muuttui vieroituksessa (6–12 kk) aikuisten kaltaiseksi. Vieroituksen jälkeen ruokavalio oli keskeinen pikkulasten suoliston mikrobiston muokkaaja. Toinen tutkimus osoitti että maantieteellinen sijainti muodosti merkittävän ympäristö- ja ravitsemustekijän: Kiinasta ja kahdesta Malesian kaupungista kotoisin olevien kiinalaisten ja malaiji-lasten mikrobiston havaittiin jakaantuvan *Bacteroides*- ja *Prevotella*-klusteriin. Kolmas tutkimus osoitti Singaporen nuorten aikuisten suoliston mikrobiston jakautuvan kolmeen klusteriin (*Bacteroides*, *Prevotella* ja *Blautia*) ja ruokailutottumukset vaikuttivat merkittävästi mikrobiston kehittymiseen. *Blautia*-klusteri (assosioitui satunnaiseen länsimaisten ruokien käyttöön) vaikutti seerumin sytokiinivasteisiin. Suoliston mikrobiston enterotyyppi ei muuttunut tutkimuksessa, jossa nuoret nauttivat probioottisella laktobasillilla fermentoitua maitojuomaa. Neljäs osatutkimus paljasti *Bacteroides*-bakteerien määrä singaporelaisten aikuisten suolistossa vähenevän merkittävästi ateriaan liitetyn polyfenolipitoisen mausteseoksen nauttimisen jälkeen. Erityisesti bifidobakteerien määrä lisääntyi suurten mausteannosten jälkeen.

Väitökirjatutkimuksen perusteella imeväisten, lasten ja aikuisten suoliston mikrobiston kehityksen tarkempi tunteminen ja koostumuksen muokkaaminen hyödyllisillä mikrobeilla (probiooteilla) ja polyfenoleilla mahdollistaa mikrobistoa muokkaavia ruokien kehittämisen ja ruokailutottumuksien seurannan. Nämä muutokset voivat edistää terveyttä.

AVAINSANAT: *Bifidobakteerit*, *Prevotella*, suoliston mikrobisto, terveellinen ruokavalio

Table of Contents

Abbreviations

ANOSIM	Analysis of similarities
ANOVA	Analysis of variance
AUC	Area under the curve
BB	*Bacteroides-Bifidobacterium*
B-C	Bray-Curtis distance
BF	Faecal samples of children
BM	Breast milk samples of mothers
BMI	Body mass index
BSA	Bovine serum albumin
BSH	Bile salts hydrolases
BV	Bacterial vaginosis
CA	Cholic acid
CA	Cinnamic acid
CDCA	Chenodeoxycholic acid
CH	Calinkski-Haravasz
CN	Southern Chinese
CS	Caesarean section
D0C	Dose 0 control
D1C	Dose 1 curry
D2C	Dose 2 curry
db-RDA	distance-based redundancy analysis
DCA	Deoxycholic acid
DSRB	Domain-specific research board
GAE	Gallic acid equivalent
GBS	Group B *Streptococcus*
GCA	Glycocholic acid
GCDCA	Glycochenodeoxycholic acid
GDCA	Glycodeoxycholic acid
GDM	Gestational type 2 diabetes mellitus
GLCA	Glycolithocholic acid
GM-CSF	Granulocyte-macrophage stimulating factor

GUDCA	Glycoursodeoxycholic acid
GZ	Guangzhou city
HALE	Heathy life expectancy
hCG	Human chorionic gonadotrophin
HDCA	Hyodeoxycholic acid
HFD	High-fat diet
HLA	Human leukocytes antigen
HMOs	Human milk oligosaccharides
IAP	Intrapartum antibiotics prophylaxis
IBD	Inflammatory bowel disease
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRB	Institutional review board
ISAPP	International Scientific Association for Prebiotics and Probiotics
JSD	Jensen-Shannon distance
KL	Kelantan city
LCA	Lithocholic acid
LcS	*Lactobacillus casei* Shirota
LcZ	*Lactobacillus casei* Zhang
LNT	lacto-N-tetraoses
MF	Faecal samples of mothers
ML	Malay
NAFLD	Non-alcoholic fatty acid
NK	Natural killer
OTU	Operational taxonomic units
P	*Prevotella*
PAA	Phenylacetic acid
PAM	Partitioning around medoids
PBS	phosphate-buffered saline
PCA	Principal component analysis
PCoA	Principal coordinate analysis
PERMANOVA	Permutational multivariate analysis of variance
PMSF	Phenylmethylsulfonyl fluoride
PN	Penang city
PROM	Preterm labour induced by rupture of membrane
QIIME	Quantitative insights into microbial ecology
RSs	Resistant starches
SCFAs	Short-chain fatty acids
SD	Standard deviation

SG	Singapore
Si	Silhouette coefficient
TCA	Taurocholic acid
TCDCA	Taurochenodeoxycholic acid
TDCA	Taurodeoxycholic acid
TGF	Transforming growth factor
Th	T helper cells
TLCA	Taurolithocholic acid
TLR	Toll-like receptor
TMCA[a+b]	alpha- and beta-tauromuricholic acid
TNF	Tumour necrosis factor
TPE	Total polyphenol excretion
TUDCA	Tauroursodeoxycholic acid
UDCA	Ursodeoxycholic acid
UF	Unknown family
UG	Unknown genus
VG	Vaginal delivery
VS	Vaginal swab samples of mothers

List of Original Publications

This dissertation is based on the following original publications referred to in the text by their Roman numerals (I–IV). The original publications have been reproduced with the permission of the copyright holders.

I Khine, W. W. T., Rahayu, E. S., See, T. Y., Kuah, S., Salminen, S., Nakayama, J., Lee, Y-K. (2020). Indonesian children faecal microbiome from birth until weaning was different from microbiomes of their mothers. *Gut Microbes*, 12(1),1761240. doi: 10.1080/19490976.2020.1761240.

II Khine, W. W. T., Zhang, Y., Goie, G. J. Y., Wong, M. S., Liong, M., Lee, Y. Y., Cao, H., Lee, Y-K. (2019). Gut microbiome of pre-adolescent children of two ethnicities residing in three distant cities. *Scientific Reports*, 9, 7831. doi: 10.1038/s41598-019-44369-y.

III Khine, W. W. T., Teo, H. T. A., Loong, W. W. L., Tan, J. H. J., Ang, G. H. C., Ng, W., Lee, C. N., Zhu, C., Lau, Q. C., Lee, Y-K. (2021). Gut microbiome of a multiethnic community possessed no predominant microbiota. *Microorganisms*, 9(4), 702. doi:10.3390/microorganisms9040702.

IV Khine, W. W. T., Sumanto, H., Loi, S. D., Lee, Y-K. (2021). A single serving of mixed spices alters gut microflora composition: a dose-response randomized trial. *Scientific Reports*, 11, 11264. doi:10.1038/s41598-021-90453-7.

1 Introduction

The human Microbiome is the most complex system in the human body, where the microbial communities coevolve with the host from birth and early life. The microbiome's diversity, profile, and composition change progressively with age (Hopkins et al., 2001, Yatsunenko et al., 2012, Odamaki et al., 2016, Buford 2017, Stewart et al., 2018, Vatanen et al., 2018) from infancy to childhood, puberty to adulthood, and older age. Early life is a critical period for developing the host, the microbiome, and human health in the later stages of life (Maynard et al., 2012, Gensollen et al., 2016). The establishment, maturation, and adaptations of gut microbiota in different age groups are influenced by diet, lifestyles, environment, and external mediation such as medicines, surgery, and so on (Yatsunenko et al., 2012, Goodrich et al., 2014, David et al., 2014, Moeller et al., 2016, Milani et al., 2017, Palleja et al., 2018).

Although it is unclear how the early life colonisers in humans are selected, they mainly transfer from the vertical transmission from the maternal microbiota. Some research reported that the perinatal microbiome exists (DiGiulio et al., 2012, Aagaard et al., 2014, Perez-Muňoz et al., 2017), while others argued that the uterine cavity is sterile (Lauder et al., 2016, Blaser & Dominguez-Bello 2016, Hornef and Penders 2017, Leiby et al., 2018). However, the infant's gut microbiota determinants during and after delivery are gestational age, intrapartum antibiotics prophylaxis, diseases, modes of delivery, types of feeding/diet, and environment. The gut microbiota of infants gradually changes to the adult-type microbiota after the weaning period. As dietary habits mainly shape the gut microbiota of children and adults, dietary interventions are essential in human gut microbiome studies relating to diet. Probiotics administration in food or as supplementary affects the basal gut microbiota of adults differently and benefits gastrointestinal health. Moreover, culinary spices and herbs are well-known for their polyphenols on health benefits, and their consumption alters the composition of the basal gut microbiota. We hypothesised that different types of food modify the basal microbiota of human in different life stages.

The thesis addresses three components of four studies: (1) composition of gut microbiota in the age range from newborn to 48 months old, school-aged (7-12 years

old), adults (18-40 years old); (2) the major determining factor on their gut microbiota; (3) their gut microbiota changes after an intervention with a probiotic fermented drink and a dietary serving with mixed spices. These intensive studies aimed to understand (1) the composition of the faecal microbiota of children (from neonates to school-aged) linked with their mothers' microbiota across different ages and compared their faecal microbiota in different ethnicities and residing cities, (2) the association between their faecal microbiota and the consumed food groups, and (3) the modification of basal faecal microbiota of adults in the multi-ethnic dietary habits of their community after introducing a probiotic fermented drink and a meal containing mixed spices in different doses.

The taxonomical names of some of the species of *Lactobacillus* have recently changed and the following species from the thesis should be recognised as the new taxonomical names as stated below.

Old taxonomical name	New taxonomical name
Lactobacillus casei Shirota	Lacticaseibacillus casei Shirota
Lactobacillus casei Zhang	Lacicaseibacillus casei Zhang
Lactobacillus vaginalis	Limosilactobacillus vaginalis
Lactobacillus murinus	Ligilactobacillus murinus
Lactobacillus rhamnosus GG	Lacticaseibacillus rhamnosus GG
Lactobacillus fermentum	Limosilactobacillus fermentum
Lactobacillus plantarum	Lactiplantibacillus plantarum
Lactobacillus salivarius	Lagilactobacillus salivarius
Lactobacillus paracasei	Lacicaseibacillus paracasei
Lactobacillus reuteri	Limosilactobacillus reuteri

2 Review of the Literature

2.1 Gestational (Pregnancy and foetus) period

2.1.1 Physiology during pregnancy

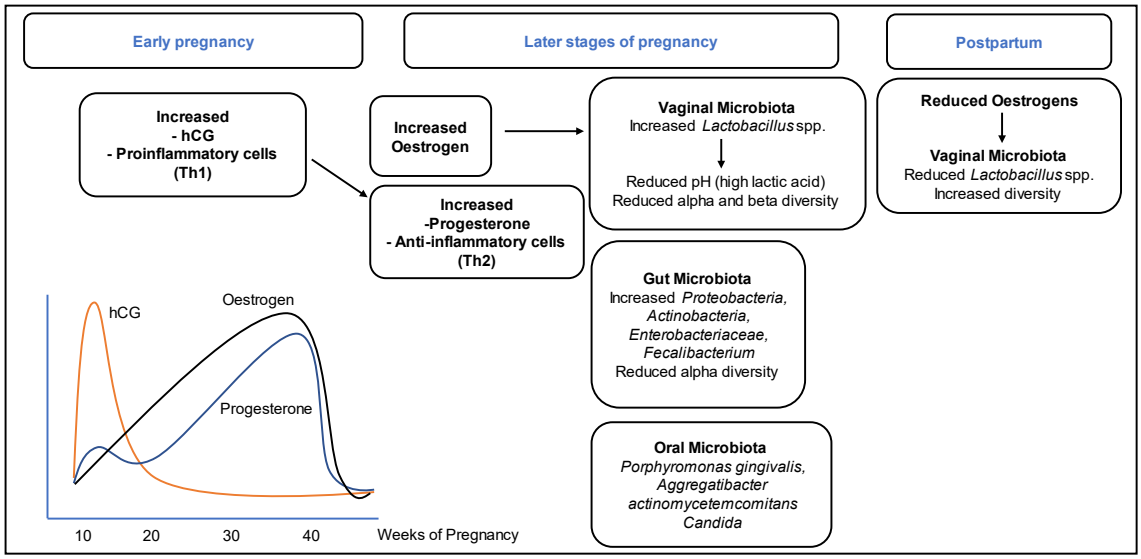


Figure 1. Hormonal and microbiota changes during pregnancy. hCG = Human Chorionic gonadotrophin, Th= T helper cells.

Healthy pregnant women undergo alterations in their physiological, anatomical, immunological hormonal and metabolic statuses (Newbern & Freemark 2011), that lead to the healthy growth and development of the infant and also support the health of mothers (Fig. 1). In addition to changes, the microbiota from different body sites of mothers, especially the gut, vagina, oral cavity, and skin, are constantly changed (Aagaard et al., 2012, Cabrera-Rubio 2012, Koren et al., 2012, Romero 2014, DiGiulio et al., 2015, Nuriel-Ohayon et al., 2016, Amir et al., 2020). Gestational gut microbiota profiles may vary for several reasons: maternal diet, geographic

variations, antibiotic usage, comorbidities, and multiple pregnancies, as well as others.

Due to the physiological changes in the first trimester of pregnancy, T helper (Th)1 cells produce pro-inflammatory cytokines (Mor et al., 2017), while the placental trophoblasts produce human chorionic gonadotrophin (hCG) hormones for placental growth (Korevaar et al., 2015). The Th1 profile then shifts to Th2 and the regulatory T cells, so that the anti-inflammatory cytokines are populated for successful implantation, and the Th2 immunological process occurs throughout pregnancy (Wegmann et al., 1993, Sykes et al., 2012). Progesterone production, which is stimulated by hCG and oestrogens, is vital in the endometrium preparation for embryo implantation (Cole et al., 2010, Berkane et al., 2017). The levels of oestrogens and progesterone start to rise during the first trimester and peak in the third trimester (Bryns 2014), while the level of hCG declines during the second trimester (Korevaar et al., 2015). During pregnancy, metabolic changes such as elevated fasting glucose levels, insulin resistance, and glucose intolerance; also occur through an increase in thyroid hormones, steroid hormones, oxytocin, prolactin, and insulin secretion (Kumar P and Morgan N 2012).

2.1.2 Maternal vaginal microbiota during pregnancy

The vaginal microbiota of low-risk pregnant women is primarily populated by lactic acids producing *Lactobacillus iners*, *L. crispatus*, *L. jensenii*, *L. johnsonii*, *L. gasseri* and *L. vaginalis* (Aagaard et al., 2012, Romero et al., 2014). During pregnancy, elevated levels of oestrogen stimulate the maturation and deposition of glycogen in the epithelium of the vagina, where lactic acids are produced by *Lactobacillus* spp. and the metabolised products of glycogen (Amabebe and Anumba 2018). Due to their anti-microbial nature and the lowered pH of vaginal surroundings, pathogens and other commensal bacteria are less abundant, and *Lactobacillus* spp. are enriched in the healthy vaginal microbiome (ÓHanlon et al., 2013, Romero et al., 2014, Amabebe and Anumba 2018). The vaginal microbiota diversity, richness (Stout et al., 2017), and composition (Romero et al., 2014) of low-risk pregnant women are more stable than non-pregnant women throughout the pregnancy (Romero et al., 2014) and during early pregnancy (Stout et al., 2017). The *Lactobacillus* spp. composition changes from one community to another (Romero et al., 2014). The type of *Lactobacillus* communities is different depending on the mother's ethnicity (Maclntyre et al., 2015, Stout et al., 2017, Serrano et al., 2019). Romero et al. stated that the composition of the vaginal microbiota varies due to pregnancy's hormonal, metabolic, and immunological effects (Romero et al., 2014). As well as, the hormonal effect on the vaginal microbiome in pregnancy, Brooks et al. revealed that contraceptives (oestrogen and progesterone) affected the vaginal microbiome with a

high abundance of lactobacilli and reduced the bacteria causing bacterial vaginosis (Brooks et al., 2017). However, the vaginal microbiota composition is more diverse during the postpartum period with the reduced counts/amounts of *Lactobacillus* spp. due to the rapid reduction of oestrogen after the delivery of the baby and placenta (Amabebe and Anumba 2018) and increased diversity compared with the pregnancy period (Maclntyre et al., 2015).

2.1.3 Maternal gut microbiota during pregnancy

In the previous literature, maternal gut microbiota was reported to significantly change during pregnancy, from the first trimester to the third trimester (Koren et al., 2012). Proteobacteria, Actinobacteria phyla, and species beta diversity among the pregnant women were profoundly increased, but species richness of alpha diversity within the samples decreased in the third trimester (Korean et al., 2012). However, another study reported that alpha and beta diversities were consistent throughout the pregnancy (DiGiulio et al., 2015). Clostridiales orders, including *Fecalibacterium* and *Eubacterium*, butyric acid producers, were raised, which is likely to prevent the rejection from the anti-inflammatory effect of the maternal immune response in the first trimester (Koren et al., 2012). In a study of the progesterone effect on gestation, the abundances of *Bifidobacterium, Neisseria, Blautia, and Collinsella* were enriched in late pregnancy (Nuriel-Ohayon et al., 2019). Koren et al. hypothesised that elevated levels of the *Enterobacteriaceae* family and *Streptococcus* in the third trimester and one-month postpartum mothers and one-month-old infants help to activate the immune system against microbes in general (Koren et al., 2012).

2.1.4 Maternal oral microbiota during pregnancy

The oral cavity, the main entrance of external bacteria from foods to the gastrointestinal tract, contains a considerable amount of microbial diversity. Early-stage pregnant Japanese women had a high total count of seven culturable bacteria from the oral cavity compared with non-pregnant Japanese women (Fujiwara et al., 2015). Two pathogenic bacteria: *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* as well as *Candida* species, were prevalent in the early pregnancy (7- 28 weeks of gestation) of the Japanese women, but Candida only in the later stage of pregnancy (17-39 weeks of gestation) (Fujiwara et al., 2015). The growth of *Candida* might be associated with increased pregnancy hormones (Fujiwara et al., 2015).

2.1.5 Effects of microbial changes in pregnancy on both mother and foetus

Mothers may encounter pregnancy-related complications such as metabolic syndrome: gestational type 2 diabetes mellitus (GDM), gestational obesity, hypertension and preeclampsia, anaemia, infections, and others. Pregnancy complications are triggered by physiological or pre-existing/external microorganisms changes. Apart from infections that have a direct effect, the remaining complications may be associated with maternal microbiomes as a direct cause or confounding factor. Alteration of the maternal microbiome subsequently shapes the composition of the infants' gut microbiome and influences the health of infants.

Many studies have shown the direct or reverse correlation between the metabolic hormone changes in pregnancy (insulin, polypeptide, Adipokine, C-reactive protein, fasting glucose, ghrelin) and specific gut bacterial taxa: *Collinsella*, *Coprococcus*, *Ruminococcaceae*, *Lachnospiraceae*, *Sutterella*, *Faecalibacterium*, *Blautia*, *Bacteroidaceae* (Gomez-Arango et al., 2016, Ferrocino et al., 2018). Koren et al. proposed that the energy extracted from foods by gut microbiota directly impacts the host's metabolism. Dysbiosis drives inflammation, weight gain, hyperglycemia, insulin resistance, and GDM (Koren et al., 2012). A study of GDM found high abundances of *Faecalibacterium*, *Anaerotruncus* species, and low abundances of *Clostridium* and *Veillonella* species in pregnant women with GDM compared to normoglycemic pregnant women in the third trimester (Crusell et al., 2018). Although weight gain in pregnancy is necessary for a normal healthy pregnancy, high amounts of microbiota differences were found in overweight pregnant women, such as *Bacteroides* and *Staphylococcus* (Collado et al., 2008) and *Blautia*, *Lachnospiraceae,* and Clostridiales (Stanislawski et al., 2017). However, low amounts of *Bifidobacterium* and *Bacteroides* (Santacruz et al., 2010) and reduced alpha diversity (Stanislawski et al., 2017) were also reported. The gut microbial composition of mothers is transferred to their infants, which consequently increases the risk of childhood obesity (Santacruz et al., 2010, Stanislawski et al., 2017). Periopathogenic organisms such as *Actinobacillus actinomycestemcomitans*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsynthia,* and *Treponema denticola* were detected in the placenta of women with pre-eclampsia which is caused by abnormal placental pathology (Barak et al., 2007). Moreover, *Bacillus cereus*, *Listeria*, *Salmonella*, *Escherichia*, *Klebsiella pneumonia*, *Anoxybacillus*, *Variovorax*, *Prevotella*, *Porphyromonas,* and *Dialister* (Amarasekara et al., 2015) were reported presenting in placental tissue samples of pre-eclampsia pregnant women.

Infections during pregnancy are common and mainly impact the foetus causing adverse effects. External infections (bacterial, viral, parasitic) enter the host through

the gastrointestinal tract (from foods), through the bloodstream, and ascending tract (from vagina and rectum) to the placenta and uterus (chorioamnionitis). Food-borne bacterial infections such as *Listeria monocytogenes*, *Salmonella enteritis*, *Campylobacter* spp., *Brucella* spp., and *Toxoplasma gondii* can cause host-related symptoms (e.g., gastroenteritis, fever, sepsis) and, premature delivery, miscarriage, stillbirth, and congenital toxoplasmosis, as well as nervous system problems (Pappas et al., 2009, Kuperman-Shani et al., 2015, Vázquez-Boland et al., 2017, Delcourt et al., 2019, Inan et al., 2019). Pathogenic bacteria or viruses may cross the maternal placenta from the haematological route to the foetus, leading to foetal death or preterm birth, which raises controversy about whether the placenta is sterile. Group B *Streptococcus* (GBS, caused by *Streptococcus agalactiae*), bacterial vaginosis (BV, dysbiosis of vaginal microbiome, caused by *Ureaplasma urealyticum* and *Mycoplasma hominis*), and urinary tract infections (caused by Gram-negative bacteria, *E. coli*, *Klebsiella pneumoniae*) are most common among reproductive women and are harmful to both mothers and infants complicating as preterm labour induced by rupture of membrane (PROM), preterm birth, bacteraemia, perinatal death and chorioamnionitis (BV) (Delzell et al., 2000, Doster et al., 2018, Rumyantseva et al., 2019). Sexually transmitted infections such as *Chlamydia trachomatis*, gonorrhea (caused by *Neisseria gonorrhoeae*), and syphilis (caused by *Treponema pallidum*) result in preterm delivery, conjunctivitis, blindness, and death (Mullick et al., 2005). Viral infections including hepatitis B, hepatitis C, human immunodeficiency virus, herpes simplex virus, cytomegalovirus, and Zika virus greatly impact the health of both mothers and infants.

Regarding premature delivery, phospholipase A2 activity which facilitates prostaglandin E2 for uterine contraction was found in *Bacteroides fragilis*, *Peptostreptococcus*, *Fusobacterium necrophorum*, *Streptococcus viridans*, *Streptococcus fecalis*, *Streptococcus* A and B, *E. coli*, *Klebsiella*, *Staphylococcus epidermidis*, *Pneumococcus*, *Lactobacillus*, and *Mycoplasma hominis* (Bejar et al., 1981). A reduced abundance of *Lactobacillus* characterises dysbiosis in the vaginal microbiome, consequently, reducing the pH of the vagina leads to vaginal infections and the production of cytokines and prostaglandins, which induce pregnancy complications such as preterm delivery. Nevertheless, preterm infants had a low species diversity and abundance of beneficial bacteria; *Bacteroidaceae*, *Bifidobacterium*, and *Lactobacillus* but high amounts of potential pathogens*; Enterobacteriaceae*, *Clostridium*, *Pseudomonas*, *Klebsiella,* and *Escherichia coli* compared to term infants (Arboleya et al., 2015, Ho et al., 2018, Liu et al., 2019). However, as preterm infants were treated with intravenous antibiotics and cared for in the neonatal intensive care unit, the microbiome's composition would be different, and it is difficult to access the direct influencing factor of the preterm gut microbiome.

2.2 Birth, neonatal, and early life period

2.2.1 Placental and meconium microbiotas

Many maternal/foetus studies have debated whether placenta and foetal tissues are sterile or in-utero colonisation by bacteria. Some findings using cultural, quantitative PCR, and metagenomic techniques have consistently pointed out that the healthy placenta and foetal tissues were detected as having specific microbial communities, such as Proteobacteria, *Fusobacterium*, *Streptococcus*, *Bifidobacterium*, *Bacteroides*, *Clostridium*, *Lactobacillus*, *Propionibacterium*, *Enterobacteriaceae* and so on (Rautava et al., 2012, Aagaard et al., 2014, Prince et al., 2016). In-utero exposure to bacteria can be seen in meconium samples of infants, in which *Staphylococcus*, *Enterobacteriaceae*, *Enterococcus*, *Lactobacillus,* and *Bifidobacterium* were predominant (Jiménez et al., 2008, Dong et al., 2015, Hansen et al., 2015, Collado et al., 2016). Compared with the faecal samples of healthy adults, meconium samples of babies of healthy mothers possessed low species diversity with high Proteobacteria and low Bacteroidetes,h however, the inter-individual variation was high (Hu et al., 2013). A recent study of bacterial seeding from mothers to infants revealed that meconium microbiota shared the most taxonomical features with the amniotic fluid microbiota from different maternal body site samples (He et al., 2020). In contrast, Perez-Muňoz et al. extensively reviewed some of the studies and concluded that more evidence is needed to prove the in-utero colonisation hypothesis, and more well-controlled methodological studies are required (Perez-Muňoz et al., 2017). Aagaard and colleagues highlighted that in-utero colonisation is possible, the low abundance and diversity of unique microbiota found may or may not be alive, and the cultivation method might be challenging.

2.2.2 Effects of maternal factors on early life microbiota

Infant microbiota especially gut microbiota is influenced by many factors such as maternal diet in pregnancy and lactation, maternal vaginal, rectal, and skin microbiotas, intrapartum antibiotics prophylaxis and antibiotics usage after birth, mode of delivery, gestational age of birth, type of infant feedings and probiotics supplementation.

2.2.2.1 Maternal diet during pregnancy

The maternal diet in pregnancy modifies the mother's microbiota composition, which is then transferred to her offspring in the womb, so it is a potent modifier of

the gut microbiome of infants, although the mechanism of transmission of microbiota is still not clear (Chu et al., 2016). Many animal studies have reported that a maternal high-fat diet (HFD) during pregnancy or lactation generally affects the health of offspring, and a maternal healthy diet may be important to the infants' health (Gawlińska et al., 2021). Another hypothesis is that the gut microbiota of infants is modified, which might also be due to the high energy intake from the HFD in the mother's milk. A primate study on high-fat diet intervention during pregnancy found that the abundance of *Campylobacter* species was persistently low in the offspring guts of HFD-exposed mothers after weaning (Ma et al., 2014). Another study of synbiotic supplementation to the offspring of HFD macaque mothers revealed either a slight or no modification of the gut microbiome with a temporary increase in *Lactobacillus* (Pace et al., 2018). During the third trimester before Caesarean section (CS) surgical delivery, a probiotic intervention was seen to modulate the immune response of the expression of the Toll-like receptor (TLR)-related gene, which expressed in the meconium samples of infants (Rautava et al., 2012).

2.2.2.2 Antibiotics usage in the perinatal period and mode of delivery

Maternal vaginal, skin or rectal microbiota may be transferred to neonates during delivery, depending on the type of delivery, such as normal vaginal or CS surgical delivery. Studies have observed that the gut microbiota composition of the vaginally delivered neonates dominated by *Bacteroides* and *Bifidobacterium* originating from the maternal vagina, is differed from that of CS-delivered infants in the first week of life (Biasucci et al., 2010, Hesla et al., 2014, Jakobsson et al., 2014, Rutayisire et al., 2016). Another study reported that *Lactobacillus*, *Prevotella,* and *Sneathia* spp. were prevalent in the meconium samples of vaginally delivered neonates (Dominguz-Bello et al., 2010). Vaginally born infants had more frequent levels of *Lactobacillus* (Kabeerdoss et al., 2013, Chu et al., 2017) but low levels of *Bacteroides* (Chu et al., 2017) compared to CS-born infants. In contrast, in the gut of CS-delivered neonates, skin bacteria such as *Staphylococcus*, *Corynebacterium*, *Propionibacterium* (Dominguez-Bello et al., 2010) or potential pathogens such as *Klebsiella*, *Enterococcus*, *Clostridium, Haemophilus,* and *Veillonella* (Helsa et al., 2014, Dogra et al., 2015, Rutayisire et al., 2016, Montoya-Williams et al., 2018) were predominant but, abundances of *Bifidobacterium* and *Bacteroides* were low. Total gut bacterial diversity was reduced in infants delivered by CS compared to those vaginally delivered (Rutayisire et al., 2016). Low bacterial diversity in infants born by CS at birth and early life leads to fewer bacteria exposure to the host immune system (West et al., 2014). Due to this hygienic hypothesis and its effect, gut microbiota dysbiosis may develop causing many medical diseases such as asthma,

allergies, obesity, celiac disease, irritable bowel syndrome and necrotizing enterocolitis (Rutayisire et al., 2016).

Mode of delivery may also determine the infants' gut microbiota; however, different co-factors are involved, mainly perinatal antibiotic administration, to determine the primary determinant of the gut microbiota of newborns. Some mothers are administered oral or intravenous antibiotics during the perinatal period, especially while giving birth, based on their underlying medical indication or for prophylactic purposes against potential infections like group B *Streptococcus* (GBS). Caesarean section delivered (emergency or elective) mothers usually receive antibiotics before surgery or at the time of umbilical cord clamping according to the guidelines of the hospital, which is called intrapartum antibiotics prophylaxis (IAP). Antibiotic-induced dysbiosis may be the primary factor influencing the infant's gut microbiota during early life.

Some papers have been published reporting that maternal IAP affects the development and composition of the gut microbiome of breastfed vaginally delivered infants with decreasing bacterial diversity and abundances of Actinobacteria, Bacteroidetes, Firmicutes, *Bifidobacterium*, *Bacteroides*, *Parabacteroides*, and increasing abundances of Proteobacteria, *Escherichia* at the first week of life (Mazzola et al., 2016, Nogacka et al., 2017). The distinct microbiome of CS-born infants in the first week of life might be changed and diversified within six weeks of life, and the mode of delivery is not a major influencer on the gut microbiota of infants (Rutayisire et al., 2016, Chu et al., 2017). Dominguez-Bello and colleagues performed the transfer of maternal vaginal fluid to their infants after being delivered by CS. Their results revealed that some vaginal bacteria were restored (Dominguez-Bello et al., 2016). The lack of vaginal microbiota could be replaced in CS-born infants before the action of an intrapartum antibiotic which has not reached the vaginal microbiome. It is also important to note that the underlying indications of CS might cause a differentiation in the microbiota of infants' gut, but further evaluation is needed to elucidate more evidence.

2.3 Infancy (First year of life)

Neonates are exposed to complex microbial communities in the surrounding environment once they are born. The infant microbiota composition and functions vary depending on complex factors: maternal factors (discussed above), infant factors (physiological, immunological development, biodata, anthropometric data, antibiotics and probiotics usage, types of feeding such as breastmilk or formula and solid foods), environmental factors (pets, air pollution, housing condition, water supply). (Fig. 2).

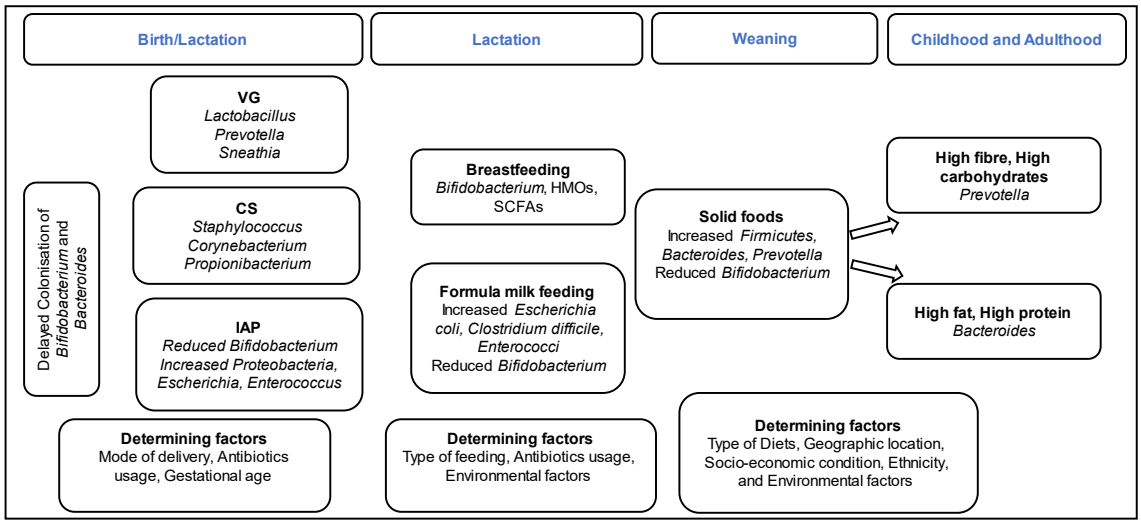


Figure 2. Determining factors and development of gut microbiota from birth to adult. VG= Vaginal delivery, CS= Caesarean section delivery, IAP= Intrapartum Antibiotic Prophylaxis, HMOs= Human milk oligosaccharides, SCFAs= Short-chain fatty acids.

2.3.1 First six months of life

2.3.1.1 Infant gut microbiota

Based on the first colonisers of gut microbiota, the successful colonisation and stabilisation of the microbiota vary. The strains of microbiota in infants progressively change the gut environment from an aerobic (facultative anaerobes) to an anaerobic (obligate anaerobes) condition by consuming oxygen in the gut as energy from the first day after birth (Fig. 2) (Bäckhed et al., 2015, Ferreti et al., 2018). Generally, the gut microbiota processes low alpha diversity (interspecies richness), high beta diversity (heterogenicity of species), and less stability in the first months of life and this gradually becomes a high alpha diversity (more complex), low beta diversity (less heterogenicity of species) and more stable overtime during the first year of life (Bäckhed et al., 2015). Low abundance of *Bacteroides* and *Bifidobacterium* in the infants delivered by CS at birth successfully colonise in the gut after four months (Bäckhed et al., 2015) to six months for *Bacteroides* (Yassour et al., 2016) and 12 months for *Bacteroides* (Jakobsson et al., 2014, Wampach et al., 2017).

2.3.1.2 Human breast milk, Human Milk Oligosaccharides (HMOs) and breast milk microbiota

Human milk is nutritious for infants, as it contains proteins, lipids, carbohydrates, lactose, water, vitamins, immunoglobulins, oligosaccharides, growth factors, cytokines, chemokines, hormones, immune cells, and microorganisms. Exclusive feeding by breast milk is recommended for at least the first six months of an infant's age and benefits the development of infants and their immune system, overall health, and protection against pathogens. Among the compounds in human milk, human milk oligosaccharides (HMOs) are well known due to their beneficial health effect on infants. They are indigestible carbohydrates and categorised as prebiotic. They consist of five monosaccharide glucose: glactose, N-acetylglucosamine, fucose, and sialic acid N-acetyl-neuraminic acid. Their primary structures are mono- and di-fucosyllactoses, lacto-N-tetraoses (LNT), lacto-N-hexaoses and lacto-N-octaoses in human milk and there are several profiles of HMOs (Bode 2009). Based on genetic loci, mothers carry different characteristics of HMOs such as secretor and Lewis blood type. Generally, the HMO profile is classified as Lewis-positive, Lewis-negative secretors and Lewis-positive, Lewis-negative nonsecretors, which contribute to the consistency of the milk. Therefore, human milk components and their microbiome vary based on maternal diet, genetics, health status, maternal obesity, and more (Lyons et al., 2020). HMOs resist gastric acidity and reach the colon, where they can be fermented by a specific type of intestinal bacteria in infants, although infants cannot degrade them directly. HMOs also have antimicrobial and antiadhesive properties (Bode 2009, Bode 2012). Commonly found in breast milk microbiota are skin and oral cavity associated bacteria such as *Staphylocccus, Veillonella, Leptotrichia, Prevotella*, lactic acid bacteria such as *Streptococcus, Lactobacillus, Lactococcus, Bifidobacterium, Leuconostoc, Enterococcus, Weissella* and others (Moossavi et al., 2019, Lyons et al., 2020). Although *Staphylococcus* and *Streptococcus* are predominantly found in human milk studies, *Bifidobacterium* and *Staphylococcus* species have been documented as species transmitted from mothers (Benito et al., 2015, Milani et al., 2015).

2.3.1.3 Effects of breast milk and formula milk feeding on infant gut microbiota (HMOs)

Bacteroides and *Bifidobacterium* are the most dominant gut microbiota in normal vaginally delivered breastfed infants in the first year of life (Bäckhed et al., 2015, Wampach et al., 2017, Laursen et al., 2017). Infantile strains of *Bifidobacterium* spp. such as *Bifidobacterium longum*, *B. breve*, *B. bifidum,* and *B. infantis* could ferment the HMOs from human milk (Bäckhed et al., 2015, Katayama et al., 2016, Stewart et al., 2018). Particularly, *Bifidobacterium longum* subsp. *infantis* could effectively

degrade HMOs and other *Bifidobacterium* spp. grow slower in HMOs (Asakuma et al., 2011, Rockova et al., 2012). Degrading small sugars become the energy source for other gut bacteria such as lactate-producing bacteria such as *Staphylococcus*, *Streptococcus,* and *Enterococcus* (Bode et al., 2012). In this way, *Bifidobacterium* provides the nutrients for other commensal gut bacteria. HMOs have bifidogenic properties, which in turn promote the growth of some *Bifidobacterium* spp. in the infant's gut (Bouhnik et al., 1999, Katayama et al., 2016, Guarino et al., 2020). At the time of fermentation of HMOs by *Bifidobacterium*, short-chain fatty acids (SCFAs) are produced in the gut (Matsuki et al., 2016). SCFAs are involved in the regulation of the innate and adaptive immune cells as well as the mucosa regulatory system of the intestine by providing the energy source for the colonocytes, which tighten the intestinal barrier and decrease the pH of the colon so that ultimately, they help to prevent potential pathogens (Hemalatha et al., 2017, ÓNeil et al., 2017, Li et al., 2018). Thus, breastfeeding before solid foods introduce around six months primarily protects the infants from potential pathogens by means of the beneficial components of breast milk and the first gut coloniser *Bifidobacterium*.

Bacteroides thetaiotaomicron, B. fragilis and B. uniformis facilitate the digestion of milk HMOs (by mucus utilisation) which *Bifidobacterium infantis* cannot use (Marcobal et al., 2011, Yassour et al., 2018). Human milk and maternal gut microbiomes have less abundance of *Bifidobacterium* spp., but it occupies the gut of the healthy infants born vaginally. This could be explained by the bacteria transferred from mothers perhaps not being selected during the breastfeeding period whereas *Bifidobacterium* and *Bacteroides* spp. are selected due to mainly HMOs and their bifidogenic properties. Yassour and colleagues also observed that secondary strains, processing a starch utilisation gene, were identified in infants (Yassour et al., 2018).

On the other hand, the gut microbiota of formula-fed infants is prominent with potentially pathogenic bacteria such as *Escherichia coli, Clostridium difficile, Enterococci*, with high levels of bacterial diversity and low amounts of *Bifidobacterium* (Bäckhed et al., 2015, Stewart et al., 2018). The research group of Ma detected less *Bifidobacterium* and *Bacteroides*, and higher *Enterococcus, Lactobacillus, Akkermansia,* and *Blautia* in the gut microbiota of the formula-fed infants compared to the breastfed infants (Ma et al., 2020). Recently, infant milk formula was improved by the addition of HMOs and prebiotics, which endeavours to promote the beneficial bacteria, bifidobacteria, but their bifidogenic effect needs to be verified.

2.3.1.4 Effects of bile acids on infant gut microbiota

Bile acids are necessary for the digestion and absorption of lipids from foods/milk. Primary bile acids (cholic acids: CA and chenodeoxycholic acid: CDCA) are

synthesised in the liver from cholesterol, and the conjugated primary bile acids are secreted into the small intestine via the bile duct, where deconjugation of conjugated primary bile acids occurs via the enzymatic reaction of bacterial bile salts hydrolases (BSH). Gram-positive Firmicutes, including *Bifidobacterium, Clostridium*, *Lactobacillus,* and *Listeria* (Tanaka et al., 2000, Ridlon et al., 2006, Jarocki et al., 2013, Gèrard 2014) and Gram-negative *Bacteroides* (Stellwag & Hylemon 1976, Yoon et al., 2017, Yao et al., 2018) show the activity of BSH. In the large intestine, deconjugated primary bile acids are converted into secondary bile acids (deoxycholic acid: DCA and lithocholic acid: LCA) through dehydroxylation by microbial 7-alpha-hydroxylase, oxidation, and epimerisation with the help of gut anaerobes such as *Bacteroides, Clostridium, Eubacterium, Lactobacillus* and *Escherichia* (Ridlon et al., 2006, Gèrard 2014, Yeo et al., 2018). In the terminal ileum, most unconjugated bile acids such as CA, CDCA, DCA are reabsorbed and transported to the liver via enterohepatic circulation, while most LCA is excreted in the faeces. Moreover, infantile strains of *Bifidobacterium* and *Bacteroides* could survive among bile acids in the gut, but it depends on the concentration of bile acids in the guts of adults (Kurdi et al., 2006, Watanabe et al., 2017, Tian et al., 2020) and the neonates' stool (Mehdi et al., 2015). Wang et al. reported that *Prevotella copri* is associated with the primary bile acids whereas *Bacteroides uniformis* and *B. xylanisolvens* are linked with the secondary bile acids (Wang et al., 2021). Taurine conjugated bile acids are prevalent in neonates due to their storage in the liver before birth, but adult-type glycine conjugated bile acids are gradually produced when solid foods are introduced (Hardison 1978, Karpen & Karpen 2017). Secondary bile acids are prominent in infants at one to two years old (Chen et al., 2019, Tanaka et al., 2020, Faden et al., 2021). Dysbiosis can also disrupt the bile acids metabolism, which is linked to cholesterol biosynthesis and hepatic and intestinal metabolism; therefore, it influences hepatic and intestinal health and the risks of cytotoxicity and carcinogenesis.

2.3.2 Weaning period and beyond six months of age

2.3.2.1 Infant gut microbiota

After six months of successful breastfeeding, semisolid foods followed by solid foods are introduced to the infants, while a mixed feeding of milk (breastmilk or formula milk) and solid foods are consumed by some infants. The weaning period is an essential milestone for infants when most changes such as cessation of breastfeeding, the introduction of solid foods, alteration in gut microbiota profiles, maturation of immunity, and metabolism occur. The gut microbiota profiles of healthy infants vary according to the types of foods introduced, the duration of milk

feeding, and other factors. The bacterial composition and diversity start to increase during the weaning period (transition from milk-based feeding to adult-type foods) (Fig. 2) (Savage et al., 2018) and approach those of the adults (Yatsunenko 2012). Upon the introduction of foods, the prominent milk-associated bacteria *Bifidobacterium* spp. is shifted to Bacteroidetes or Firmicutes dominant communities (De Filippo et al., 2010, Fallani et al., 2011, Vallès et al., 2014).

Along with the phylum shift, the levels of SCFAs also increase, which is associated with the SCFAs producing bacteria like Bacteroidetes (Koenig et al., 2011). The profiles of bacteria start to change around six months of age when complementary solid foods are introduced, irrespective of the mode of delivery, and most of them stablise at one year of age. The antibiotic treated groups had a less diverse and stable gut microbiome. Yassour et al. proved that three families of *Bacteroidaceae, Ruminococcaceae, and Lachnospiraceae* were in low abundance before one year, then raised and stabilised after one year, while the other three families of *Bifidobacteriaceae, Clostridiaceae* and *Enterobacteriaceae* had the opposite trend (Yassour et al., 2016). As foods enriched in carbohydrates, fibres, and proteins are introduced, the functional genes associated with starch, carbohydrates, pyruvate metabolisms, vitamin biosynthesis, and xenobiotic degradation are upregulated (Yatsunenkoet al., 2012, Bäckhed et al., 2015, Laursen et al., 2017). Within five days of changing from breast milk to cows milk during weaning onto solid foods rapidly increases the amounts of *Bacteroides, Blautia, Parabacteroides, Coprococcus, Ruminococcus, and Oscillospira* spp. but, reduces *Bifidobacterium, Lactobacillus and Escherichia, and Clostridium* spp. (Davis et al., 2016). This longitudinal study revealed that without breastfeeding the gut microbiome profile changes in a short time.

2.3.2.2 Immunity and infant gut microbiota

The immune system of infants is not as mature as adults but develops over time from birth to 2-3 years of age under the hygiene hypothesis (Martin et al., 2010). Newborns rely mainly on the innate immune system to defend against external microbes. The innate immune system involves epithelial (skin) and mucosal (intestine) cells such as antigen-presenting cells, natural killer (NK) cells, dendritic cells, neutrophils, mast cells, eosinophils, cytokines, chemokines, peptides, the complement cells, and others. The adaptive or acquired immune system comprises B and T lymphocytes which further differentiate into T helper (Th) Th1 (cell-mediated response), Th2 (humoral and allergic responses), or Th17 (regulation). Those Th cells respond to the specific stimuli according to activation of the type of cytokines. Apart from major infections, the response of healthy infants' immune system generally activates at birth, during breastfeeding, and also the vaccination and weaning periods (Georgountzou and

Papadopoulos 2017). Among the cytokines, T, B regulatory cytokines such as Interleukins (IL)-10, -35 and TGF-β and Th2-derived cytokines such as IL-4, -5, -13 participate in the anti-inflammation process, whereas Th1, Th9, Th17, Th22, DC, macrophages, neutrophils, and more cells produce pro-inflammatory cytokines. Several studies have shown the association between gut microbiota and inflammatory diseases or allergic reactions (Martin et al., 2010). *Bifidobacterium bifidum* and bifidobacteria spp. enhanced the production of anti-inflammatory cytokine IL-10 (Sun et al., 2020, Hong et al., 2021), whereas *Bacteroides fragilis* and *B. ovatus* also prevented lipopolysaccharide-induced inflammation via the Th17 pathway (Tan et al., 2019). Tissue cultures and mice studies have shown that *Bacteroides distasonis*, *Clostridium clostridioforme*, *Lactobacillus acidophilus*, and *L. murinus* and SCFA metabolite, i.e. butyric acids from butyrate-producing bacteria such as clostridia induced Treg cells which are involved in colonic inflammation and allergies (Geuking et al., 2011, Furusawa et al., 2013).

2.3.2.3 Non-communicable diseases and infant gut microbiota

The gut microbiota affects immune regulation and development and vice versa. Dysbiosis might lead to immune-related disorders such as allergies, atopic dermatitis, asthma, autoimmune diseases, inflammatory bowel disease (IBD), celiac diseases, necrotizing enterocolitis, diabetes, and obesity. Numerous studies around the world have shown that infants with risks of allergies, atopic dermatitis, and asthma have a different set of gut microbiota commonly indicated by, low levels of *Bifidobacterium* and *Bacteroides, Lachnospira, Veillonella, Faecalibacterium,* and *Rothia* and a high abundance of *Enterobacteriaceae* (Kalliomäki et al., 2001, Björkstén et al., 2001, Abrahamsson et al., 2012, Azad et al., 2015, Arrieta et al., 2015). Early life dysbiosis is associated with the risk of the development of asthma at seven years of age (Abrahamsson et al., 2012). Reduced total bacterial diversity and a high abundance of *Enterobacteriaceae* and *Clostridium* spp. were found in Japanese infants with food allergies (Tanaka et al., 2017). Gut barrier permeability is reduced in type 1 diabetes patients due to gut bacteria dysbiosis (Pellegrini et al., 2017), and they have fewer genes for the fermentation production of SCFAs (Wen et al., 2008). Non-breastfed infants from Norway and Denmark had a profile of a higher risk of type 1 diabetes than breastfed infants, and the duration of breastfeeding is not related to the risk (Lund-Blix et al., 2017), but the timing of changing to solid foods is the determinant and 4-5 months of age is recommended (Frederiksen et al., 2013). The gut microbiome of an autoimmune disease such as Coeliac suffering infants possessed reduced *Bifidobacterium* and increased *Bacteroides* levels (Collado et al., 2009) and a higher abundance of Firmicutes and Proteobacteria.

Infants with a lower abundance of Actinobacteria had a higher genetic/human leukocyte antigen (HLA) risk of Coeliac disease (Olivares et al., 2015).

2.4 Toddlers (one to four years of age)

2.4.1 Diet and gut microbiota

The toddler period is another milestone for babies, in which they are more exposed to the environment than previously more than before; thus, unfortunately, illnesses and infections are relatively high at this age (Fig. 2). Danish toddlers had a high abundance of *Lachnospiraceae, Ruminococcaceae, Eubacteriaceae, Rikenellaceae*, and *Sutterellaceae* but a low amount of *Bifidobacteriaceae, Actinomycetaceae, Enterobacteriaceae, Lactobacillaceae, Veillonellaceae, Enterococcaceae,* Clostridiales*, Carnobacteriaceae*, and *Fusobacteriaceae*; and maternal obesity was not observed to influence toddlers' gut microbiota during weaning (Bäckhed et al., 2015, Yassour et al., 2016, Laursen et al., 2017). Different types of bacteria depend on the types of food consumed. Fibre enriched foods favour *Pasteurellaceae, Prevotellaceae, Veillonellaceae, Eubactericeae*, and *Fusobacteriaceae,* whereas protein intake associates with *Lachnospiraceae, Clostridiaceae, Ruminococcaceae, Erysipelotrichaceae, Peptostreptococcaceae*, and *Sutterellaceae* and breastmilk bacteria such as *Bifidobacteriaceae, Enterococcaceae,* and *Lactobacllaceae* were negatively related to the fibre or protein intake (Laursen et al., 2017). Almost undetectable amounts of the beneficial bacteria, *Akkermansia muciniphila* (a mucin-degrading) and *Faecalibacterium prausnitzii* (a butyric acid, SCFA- producing) in early life, increased up to 2 and 3% of total bacteria at two to three years of age (Yassour et al., 2016). In the Australian children's study, dairy intake, vegetable intake, soy/pulse/nut intake had positive correlations with the relative abundance of *Erysipelatoclostridium spp., Lachnospira, Bacteroides xylanisolvens*, respectively, and but fruit intake was associated with the relative abundance of *Ruminococcus gnavus* (Smith-Brown et al., 2016).

2.5 Childhood and adolescent hood

2.5.1 Effect of diets, geographic location, socio-economic status, ethnicity, and gut microbiota

Gut microbiota composition and functions also varied depending on several complex factors: host/child factors (type of diets, biodata, anthropometric data, antibiotics, and probiotics usage) and environmental factors (geographical location, socio-

economic condition, living environment, pets, air pollution, sanitation, hygiene, water supply and more) (Fig. 2). Here, the effects of diet, geographical locations, and ethnicity on the gut microbiota of children are the focus of the discussion. Different geographic locations cover factors such as dietary habits, socio-economic status, lifestyle, culture, and living environments. Southeast Asia is rich in culture and diets, and each country possesses its own unique heritage. Regarding food culture, Indonesians eat more carbohydrates (Indica rice) and vegetables, and they have unique cuisine (e.g., Indonesian fried chicken, Nasi goreng). Singapore is a multi-ethnic, multicultural global clean city where people can enjoy all the available worldwide cuisine (Naidoo et al., 2017). They tend to have different types of foods/cuisines every day because food courts can be found every corner, and food prices are affordable.

Modernisation in developing countries has changed dietary habits and this has shaped the gut microbiota composition. In the comparison of gut microbiota of children from five different Asian countries, *Bacteroides-Bifidobacterium* (BB) was the dominant group found in China, Japan, and Taiwan, but *Prevotella* (P) was the dominant groups clustered mainly in Thailand and Indonesia, where children consumed more resistant starch (Nakayama et al., 2015). Chinese children from Beijing and Lanzhou cities had only the BB type (Nakayama et al., 2015). Similar bacterial clusters were found in the Philippine cohort of urban (BB) and rural (P) cities. BB-type bacteria expressed sugars, amino acids, and lipids metabolism due to the high-fat diet consumption, while P-type bacteria activated the digestion of the complex carbohydrates (Nakayama et al., 2017). Children living in urban areas (Bangkok) had more Actinobacteria, Bacteroidales, and Selenomadales and fewer Clostidiales and bacterial diversity but rural children in Buriam had higher Clostridiales, *Ruminococcaceae, Peptostreptococcaceae,* and butyric and propionic acids because urban and rural children ate high-fat and vegetables-based diets, respectively (Kisue et al., 2018).

Prevotella_9 and *Bacteroides* dominant groups were reported among Han and non-Han Chinese from different regions of China and the conclusion was that geographical location-related factors affect gut microbiota composition and diversity more than ethnicity factors (Lin et al., 2020). Comparing the ileal bacteria compositions of Caucasians and Chinese from Australia and Chinese from Hong Kong, the healthy children's microbiotas were similar (Prideaux et al., 2013). Nevertheless, it was found that the different ethnicities from different regions (urban and rural) of China had nine bacteria genera containing SCFA producers (*Prevotella* was not included) differentiated by the children's ethnicities/geographic locations and lifestyles (Zhang et al., 2015).

Moreover, *Prevotella, Butyrivibrio*, and *Oscillospira* with few *Bacteroides* were enriched in Bangladeshi children's gut microbiota compared to USA children, but

their bacterial profile changes are not as stable as in US children. Bangladeshi ate less refined sugar enriched foods and meat, but their foods were rich in rice, bread, and lentils (Lin et al., 2013). Although it cannot be excluded that geography or ethnicity factors determine the gut microbiota of children, the dietary differences can be delineated. Another study on the differences in dietary habits also showed that the Mediterranean diet of Egyptian children enriched the *Prevotella* group, overexpressed polysaccharide-degrading genes, and had high fecal SCFAs compared with USA children (Shankar et al., 2017). Three bacterial clusters (*Bacteroides*, *Bifidobacterium,* and *Prevotella*) were dominant in Dutch school-age children. Dietary fibre and plant-based protein factors of *Bacteroides* and *Prevotella* were differentiated from the *Bifidobacterium* cluster solely due to breastfeeding duration in early life. Other factors such as maternal BMI, birth weight, and breastfeeding duration contributed to the *Bacteroides* cluster (Zhong et al., 2019).

Studies thus far suggest that environmental factors such as diets, geography, socioeconomic status, and hygienic practices, but not ethnicity, affect the gut microbiota. A study performed in Malaysia reported that the gut microbiome of different ethnic groups with high socio-economic status (Malay and Chinese) was similar but distinct from that of the tribe: Orang Asli. Sugar and fat metabolism were overexpressed in Malay and Chinese children (Chong et al., 2015). De Filippo and colleagues compared the gut microbiota of Italian and African tribes: Burkina Faso children also proved that modernised and rural diets influence the gut microbiota. Burkina Faso children consumed a traditional high-fibre diet containing cereals (millet and sorghum grains), legumes, and vegetables and lived in clay-made houses. Their gut microbiota was enriched in Bacteroidetes, *Prevotella*, *Xylanibacter,* and *Treponema,* which produces SCFAs, whereas Italian children had more Firmicutes and consumed high fat, protein, and sugar with less fibre (De Filippo et al., 2010). Regarding the impact of the genetic factor on the gut microbiome, twins/triplets (monozygotic and dizygotic) studies born by CS concluded that genetic factors might be a minor influence on the gut microbiota (Murphy et al., 2015, Yang et al., 2020). The environmental factor is the major determinant of gut microbiota at 12 months of age, and the postnatal antibiotic effect disappeared from month two and was stable at 12 months (Murphy et al., 2015).

The same living environment also contributes to similar gut microbiota. A huge sample size study investigating the genetic factor influencing the gut microbiome revealed that genetics is not significantly associated with the gut microbiome; however, a comparable bacterial composition was found in adults who are not genetically related but shared the household. Inter-individual variations were related to diet, drugs, and other factors (Rothschild et al., 2018). In a mice study of animals living in the same cage, similar bacterial communities were observed in the same

cages but not another cages, even though the same donor mice's colonic contents were inoculated (Lungberg et al., 2017).

Bacteroides genera are usually friendly commensals found in the general population, mainly in children, but they also have opportunistic harmful effects like the production of toxins and antimicrobial proteins and also competition for nutrients in the gut. A high abundance of *Bacteroides* leads to leaky gut inflammation and subsequently to colorectal carcinogenesis and autoimmune inflammatory cardiomyopathy through the proliferation of IL-8 inflammatory cytokines. However, the overactivated inflammatory responses prevent bacterial and viral infections. Ironically, some *Bacteroides* genera also produce SCFAs, and it has been reported that they have anti-inflammatory effects which reduce inflammation in the whole body (Zafar & Saier 2021). *Prevotella* species are also SCFA producers, and have health benefits such as reducing inflammation, obesity, lowering cholesterol, and more. On the other hand, this type of dysbiosis can become harmful, leading to obesity, diabetes, hypertension, non-alcoholic fatty liver disease (NAFLD), and more (Precup & Vodnar 2019). There is extensive research on the health benefits and harmful effects of *Prevotella* and *Bacteroides,* and the discrepancies could be attributed to the strain and mechanistic differences. A balanced microbiome is nevertheless necessary for maintaining a healthy human gut.

2.6 Adulthood

2.6.1 Diet and gut microbiota

Gut microbiota in adults is also varied, and factors influencing the gut microbiome are discussed in the childhood session (Fig. 2). In addition, hormonal factors also modulate the gut microbiota during the reproductive age, especially in females.

The gut microbiome composition of adults is more stable, and the diversity is higher than children. In the gut microbiota of healthy Thai adults, *Faecalibacterium prausnitzii, Blautia wexlerae, Prevotella copri, Escherichia coli, Eubacterium rectale, Bifidobacterium adolescentis, Klebsiella pneumoniae*, and other bacteria were abundant. The amount of rice intake strongly correlated with the abundance of total *Bacteroides* and *Bacteroides uniformis*. (La-ongkham et al., 2020). Compared to Malawi (rural), and the gut microbiota of Amerindian adults, the genes encoded for sugar, protein, and bile salt metabolism were overexpressed in US adults and contained more protein and fat (Yatsunenko et al., 2012). *Faecalibacterium prausnitzii*, *Bacteroides*, *Blautia*, *Dorea longicatena*, *Roseburia*, *Ruminococcus*, *Eubacterium*, and *Butyricicoccus pullicaecorum* are SCFAs producing bacteria, and *Faecalibacterium prausnitzii* were prevalent in the USA and Chinese cohort (Huse et al., 2012, Zhang et al., 2015).

Blautia is related to a high-fibre diet and is found in the gut microbiota of Austrians (Suguru et al.,2016). *Blautia* is a SCFA producer and produces bacteriocins (secondary metabolites) that suppress pathogens such as *Clostridium* and vancomycin-resistant enterococci (Caballero et al., 2017). Moreover, it can reduce the inflammation (upregulating Treg cells) caused by obesity (Benitez-Paez et al., 2020). However, it is associated with the incidence of IBD, and colorectal cancer (Chen et al., 2012). *Faecalibacterium prausnitzii* is a mucin degrading bacteria that reduces epithelial inflammation by inhibiting IL-8 secretion and related anti-colorectal carcinogenesis (Sokol et al., 2008).

2.7 Dietary supplements

Diet is one of the most important modulators of composition, diversity, and functions of gut microbiota in any age group. The modulated bacteria affect human health and diseases. Based on this knowledge, supplementing healthy diets with probiotics, prebiotics, and dietary bioactive metabolites such as phenolic compounds leads to a healthier human microbiome.

2.7.1 Probiotics and human gut microbiota

According to the consensus statement of International Scientific Association for Prebiotics and Probiotics (ISAPP) in 2015, a probiotic is defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2015). The mechanisms of probiotics are the modulation of commensal bacteria, normalisation of unbalanced bacteria, regulation of intestinal transit, prevention/competition of pathogens, colonisation resistance, acid and SCFA production, gut barrier regulation, neutralisation of carcinogens, immunological effects, endocrinological effects, neurological effects and more (Hill et al., 2015). Probiotics can be applied as dietary supplements such as dairy products, non-dairy products, drugs/powder, or external applications. Typically, lactic acid producing *Bifidobacterium* (*adolescentis, animalis, bifidum, breve* and *longum*), *Lactobacillus rhamnosus* GG*, L. casei, L. paracasei, L. acidophilus, L. johnsonii, L. fermentum, L. gasseri, L. plantarum*, and *L. salivarius*, *Streptococcus thermophilus* strains and *Saccharomyces boulardii* are used. A wide range of studies have reported the health benefits of probiotics, particularly the modulation of human gut microbiota, intestinal infections through immune activation, diarrhoea, constipation, colon cancer, intestinal colic, necrotising enterocolitis, non-communicable gut diseases, hypercholesterolemia, obesity, diabetes, and allergies (Sanders 2011). Numerous studies worldwide have reported that their beneficial effects, efficacies, survivability, and functions depend on the strains' specificity, the duration of the

therapy, basal gut microbiota, diets, etc. Here, this review focuses on the effects of a probiotic intervention on gut microbiota and the immunological effect of probiotics.

The interaction between probiotics and intestinal microorganisms is complex. *Lactobacillus casei strain* Shirota (LcS) administration for six months to Japanese children showed that *Bifidobacterium* was increased, but it reduced *Enterobacteriaceae* and *Staphylococcus* after three and six months of intervention (Wang et al., 2015). A similar study in adolescents from the Netherlands showed no differences in bacterial diversity and compositional stability after LcS administration (Manouni et al., 2019). *Lactobacillus rhamnosus* GG was introduced to children from Finland for seven months and found that the number of *Lactococcus, Lactobacillus gasseri, Ruminococcus lactaris, Prevotella melaninogenica* and *P. oralis* were higher, but *E.coli* was reduced (Korpela et al., 2016). After a probiotic *Lactobacillus casei* Zhang (LcZ) intervention in healthy adults from six Asian countries was performed, the relative abundance of *Lactobacillus*, *Roseburia*, *Coprococcus*, and *Eubacterium rectale* increased upon consumption, but potential pathogens such as *Blautia* and *Ralstonia pickettii* diminished. *Prevotella*/ *Faecalibacterium* cluster changed to *Faecalibacterium*/*Bacteroides* cluster after the consumption (Hou et al., 2020). Interestingly, Singapore samples had the lowest variability of gut microbiota throughout LcZ consumption, which led to the assumption that Singapore adults' gut microbiome is stable and resistant to colonisation by the LcZ probiotic. Regular intake of specific probiotics for a long time is essential for modifying intestinal microbiota. A LcS intervention study in Singapore with adults also showed that LcS were recovered from faeces, but it was not able to colonise after 14 days of consumption. This might be due to Singaporean adults' basal gut microbiome and dietary habits (Khine et al., 2018).

Probiotics are involved in mucosal immunity, especially in the gastrointestinal tract, with the help of IgA and cytokines. Immuno regulation by probiotics is strain specific. Gram-positive strains of the *Lactobacillus acidophilus* strain CRL 1462 and A9, *Lactobacillus casei* strain CRL431 in mice expressed toll-like receptor (TLR)-9 cells and further produced IFN-γ and TNF-α, pro-inflammatory cytokines, by lipopolysaccharides stimulation (Dogi et al., 2010). Immune regulation of probiotics includes immune-stimulation and immune-regulation with the help of cytokines. In the immune-regulation arm, IL-10 cytokines production by dendritic cells and Treg cell was stipulated, thus reducing inflammation responses (allergy, autoimmune diseases, and others). The immunostimulatory probiotics (in infection, cancer, and allergy responses) stimulate macrophages and then activate IL-12. Moreover, natural killer cells (NK) are also involved in the process resulting in Th1 cells being produced and inhibiting allergen-specific Th2 cells (Chiba et al., 2010).

Consumption of combined *Lactobacillus* and *Bifidobacterium* species in rats upregulated IL-10 and downregulated TNF-α and IL-6, implicating the

immunomodulatory effects of probiotics (Karamese et al., 2016). A probiotic mixture of *Lactobacillus paracasei* and *L. reuteri* was administered to the IL-10 deficient mice and reduced the risk of inflammatory bowel disease caused by *Helicobacter hepaticus* (Peňa et al., 2005). In tissue culture with Crohn's disease, *Lactobacillus casei* and *L. delbrueckii subsp. bulgaricus* decreased the TNF-α production (Borruel et al., 2002). In allergic and atopic diseases, the following have been reported: *Lactobacillus rhamnosus* GG consumption upregulated IL-10 production (Pessi et al., 2000), enhanced IFN-γ production (Pohjavuori et al., 2004), *Lactobacillus casei* strain Shirota in vivo study downregulated IgE production (Shida et al., 2002), and *Lactobacillus brevis* coagulans consumption suppressed IFN-α (Kishi et al., 1996). Moreover, supplementation of *Lactobacillus* GG prevented gastroenteritis (Schnadower et al., 2018), necrotising enterocolitis (Meyer et al., 2020), respiratory tract infections (Hojsak et al., 2009), urinary tract infections (Ates 2016) and reduced acute diarrhoea caused by the rotavirus (Ahmadi et al., 2015).

2.7.2 Prebiotics and human gut microbiota

The consensus statement of ISAPP in 2017 defines a prebiotic as "a substrate that is selectively utilised by host microorganisms conferring a health benefit" (Gibson et al., 2017). The features of prebiotics are (1) resistance to gastric acidity and digestive enzymes and gastrointestinal absorption, (2) fermentation/utilisation by the host's gastrointestinal bacteria (e.g., *Bifidobacterium*), and (3) stimulation of gastrointestinal bacteria growth by promoting the host's wellbeing and health. (Gibson et al., 2004). Human milk oligosaccharides are well-known prebiotics and have been briefly described in the previous "sections 2.3.1.2 and 2.3.1.3". Other natural prebiotics can be found in fruits such as tomatoes, bananas, berries, garlic, onions, legumes, green vegetables, oats, barley, wheat, etc. Naturally occurring compounds with prebiotic properties are inulin, lactulose, lactitol, fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), isomalto-oligosaccharides, xylo-oligosaccharides (XOS), mannanoligosaccharides (MOS) and other oligosaccharides, phenolics and phytochemicals (Crittenden et al., 2008). Prebiotic polysaccharides include inulin, resistant starches (RSs), cellulose, pectins, and other dietary fibres such as beta-glucan and guar gum. Prebiotics activate the growth and activity of favourable bacteria such as *Bifidobacterium* in the gut, leading to increasing SCFAs, reducing pH levels in the gut, and ultimately promoting intestinal health.

Resistant starches, homopolysaccharides of glucose, are mainly found in indigestible carbohydrates (RS1): whole grains, granular starch (RS2), e.g., green bananas, uncooked potatoes, maize; retrograded starches (RS3): by cooking and

cooling; chemically modified (RS4) by esterification, crosslinking and transglycosylation (Lattimer & Haub 2010). Prebiotics are now widely available in shops as supplements or foods or as applications on the skin. Martínez and colleagues observed that RS2 increased the abundance of *Ruminococcus bromii*, and *Eubacterium rectale* while RS4 promoted the abundance of Actinobacteria, Bacteroidetes, *Bifidobacterium adolescentis* and *Parabacteroides distasonis* (Martínez et al., 2010). The effects of RSs on the gut microbiome involve promoting bacterial fermentation, SCFAs production, and reduction in the formation of secondary bile acids in the gut, which may prevent colonic cancer (Hylla et al., 1998, Jenkins & Kendall 2000). They also help to reduce postprandial glucose, lower inflammation, and other health benefits (Nilsson et al., 2008, Maziarz et al., 2017). Indica rice, a resistant starch, is consumed by most Indonesians (Calingacion et al., 2014, Custodio et al., 2019).

2.7.3 Polyphenols and human gut microbiota

Polyphenols are non-nutrient secondary plant metabolites with four main subclasses: phenolic acids, stilbenoids, flavonoids, and lignans. They can all be extracted from different plants such as fruits, vegetables, nuts, spices, and beverages. Their effects and functions are as antioxidants, anti-inflammatories, and antimicrobials on specific types of gut bacteria; they also stimulate some gut bacteria, and are chemoprotective, cardioprotective and antioestrogenic (Willcox et al., 2004). While polyphenol compounds from foods modulate the gut microbiota composition, symbiotically, gut microbiota also helps to break down the compounds for absorption.

In vitro studies have shown that phenolic compounds are deconjugated by specific enzymes of some obligate anaerobic bacteria, including species of *Bacteroides, Bifidobacterium, Lactobacillus, Ruminococcus, Escherichia coli, Clostridium, Enterococcus* and *Butyrivibrio* (Andreasen et al., 2001, Couteau et al., 2001, Clavel et al., 2006, Wang et al., 2005). Recently *in vitro* and *in vivo* studies suggest that polyphenols could also be considered prebiotics that can modulate/enhance beneficial bacteria: *Lactobacillus* and *Bifidobacterium* (Larrosa et al., 2010, Dueñas et al., 2015, Lu et al., 2017). Lu and colleagues performed an intervention study with the consumption of a 5g/day dose of mixed spices (cinnamon, oregano, ginger, black pepper, and cayenne pepper) in healthy adults for two weeks. A significant reduction of Firmicutes phylum abundance increased the abundance of *Bifidobacterium, Lactobacillus,* and *Bacteroides* after consumption compared with the baseline before consumption (Lu et al., 2019). Many studies have pointed out that the Firmicutes/Bacteroidetes ratio is reduced and the gut microbiome modulated after consuming polyphenols. However, some discrepancies among the

studies are found due to other variable factors such as experiment designs, methods, host factors, environmental factors, diets, and the individual's basal gut microbiome.

Due to their bifidogenic-like and prebiotic effects, polyphenols exert potential health benefits. Polyphenol rich foods such as red wine (Clemente-Postigo et al., 2013), wild blueberries (Guglielmetti et al., 2013), tart cherries (Mayta-Apaza et al., 2018), pomegranates (Vlachojannis et al., 2015), grapeseed, wine, cocoa, green tea, and anthocyanins (Dueñas et al., 2015) may reduce the risk of obesity (Tomás-Barberán et al., 2014), colorectal cancer (Tomás-Barberán et al., 2014), lower cardiogenic diseases (González-Sarrías et al., 2017), and lower bad cholesterol (Selma et al., 2017). However, polyphenols also have antimicrobial properties, and some bacteria may vanish in rich polyphenols foods. However, vegetables, fruits, tea, and cocoa may boost specific gut microbiota (Lee et al., 2006, Massort-Cladera et al., 2012, Parker et al., 2013).

3 Aims

The presented studies aimed to investigate the composition of the gut microbiota of infants, children, and adults and to assess diet as a major determinant influencing gut microbiota in healthy children and adults.

The specific aims were as follows:

1. (a) To understand the faecal, vaginal, and breast milk microbiota profiles among Southeast Asian (Indonesian) mothers and compare the faecal microbiota of their vaginally delivered, full-term, breastfed children from birth to 48 months of age. (I)

   (b) To determine the level of the immunological markers (cytokines) and bile acids across specific ages of children and the effect of diet on the faecal microbiota after weaning. (I)

2. To evaluate the gut microbiota of children of Chinese and Malay origin residing in Penang, Kelantan, and Guangzhou, and compare the children between and within the cities, searching for factors (food types, ethnicities, and geographic locations) impacting the gut microbiome. (II)

   (a) To characterize the gut microbiota composition and cytokines levels of young adults from Singapore and the changes after the consumption of a *Lactobacillus* containing fermented milk drink. (III)

   (b) To verify if Singapore's multi-ethnic dietary practices of the frequent changes, modify the gut microbiome. (III)

3. To examine the acute, dose-dependent effects of mixed spices on the gut microbiota of Singapore Chinese male adults. (IV)

4 Materials and Methods

4.1 Study design, cohorts, and recruitment

The overviews of the studies and examined analyses are presented in figures 3-6.

The mother-child pairs study (Study I) was conducted as a cross-sectional study on the pairs from less than one month to 48 months. The participants were recruited at three community health care centres in Yogyakarta, Indonesia. Children born by normal vaginal delivery, with no history of intrapartum antibiotic prophylaxis, and no record of hospitalisation, and who were exclusively breastfed before the weaning period of 6 months old were included in the study. A total of 157 out of 300 mother-child pairs participated after the screening for the inclusion criteria. All the pairs were categorised into six groups based on the age of the child: less than one-month-old (25 pairs), one to less than three months old (10 pairs), three to less than six months old (23 pairs), six to less than 12 months old (35 pairs), 12 to less than 24 months old (26 pairs) and 24 to less than 48 months old (38 pairs).

In the cross-sectional design of the pre-adolescent children study (Study II), the healthy children aged 7-12-years-old were recruited at their schools from Guangzhou, China, Penang, West Malaysia, and Kelantan (East Malaysia). A total of 201 children: Guangzhou southern Han Chinese (n= 81), Penang Southern Han Chinese (n= 21), Penang Malay (n=21), Kelantan Southern Han Chinese (n=45), and Kelantan Malay (n=33), participated. None of the participants consumed either pre/pro-biotics for two weeks or antibiotics for three weeks prior to the study, and they had not required hospitalisation or medical attention during the previous three months.

In the fermented milk drink intervention study (Study III), 100 healthy young adults from a Singapore tertiary institution were enlisted. The study included those participants who were 18-30 years old healthy adults with a normal range of Body Mass Index (BMI) (18.5-24.9 kg/m^2), no history of gastrointestinal illness, not taking long-term medication, not planning an overseas trip, able to drink the fermented milk with *Lactobacillus* casei during the study period, able to avoid any other fermented food products during the study period, and able to provide the informed written consent. Participants who had an allergy or intolerance to a special diet, who had been using antibiotics, antimycotics, antidiarrheal, or laxative medication 30 days

prior to the study and were absent in compliance with the study protocol were excluded. After screening for eligibility, 75 participants were included in the study. Once screened, the participants had to abstain from the consumption of probiotics or fermented foods and food products except for the fermented milk products provided throughout the study period, and they had to maintain their dietary habits and lifestyle. The study was carried out over 42 days, including a 14- day baseline period (washout period), a 14-day ingestion period, and a 14-day follow-up period. During the ingestion period, participants consumed one bottle of 100 ml fermented milk containing 10 billion *Lactobacillus casei* strain Shirota (LcS) every morning for 14 days.

A total of 18 healthy Chinese adults (21-40 years old) from the National University of Singapore students' community took part in the randomised, crossover, acute, Indian spice-based intervention study (Study IV). The volunteers were included if they were 21-40 years of age with a BMI of 18.5-27.5 kg/m^2 and Chinese males who had no habit of regularly having large amounts of Indian curries, as this could prevent the potential residual effect of Indian curries and hormonal influences. Volunteers were excluded from the study if they had metabolic, cardiovascular, hepatic, and renal problems, high blood glucose (≥ 6.0 mmol/L) and blood pressure (≥ 140/90 mmHg), a waist circumference > 90 cm, had allergies to the test food or ingredients, had a smoking habit or drinking ≥ 12 units of alcohol per week, were on regular medications or dietary supplements. This study is the subset analysis of the original study of Haldar et al., 2019, and the details of the study design can be found in their paper(Halder et al., 2019). Three participants discontinued the study because they did not complete all three intervention sessions, thus, samples from 15 participants were analysed for Study IV. The study was designed to contain a three-day run-in period before each intervention session, one test meal on Day 1, three standard meals (on Day 0 evening, on Day 1 lunch and dinner, on Day 1 snack), three intervention sessions (Dose 0 control; D0C, Dose 1 curry; D1C and Dose 2 curry; D2C) and a14-day washout period between each session (their own choice of meals). The test meal breakfast on Day 1 was prepared with white rice in a dish with a mixture of seven dried spice powders at doses of 0-g (for no spices; D0C), 6 g (for low spices; D1C), and 12 g (for high spices; D2C). The mixture of spices in the D1C and D2C meals were turmeric, dried Indian gooseberry (amla), coriander seeds, cumin seeds, cinnamon, cayenne pepper, and clove at 8:4:4:4:2:1:1 ratio. The meals, ingredients, sources, cooking methods, and appliances were standardised for individual participants across all three intervention sessions. During each intervention session, all participants had to abstain from consuming spices or other polyphenol-rich foods and doing vigorous physical activity.

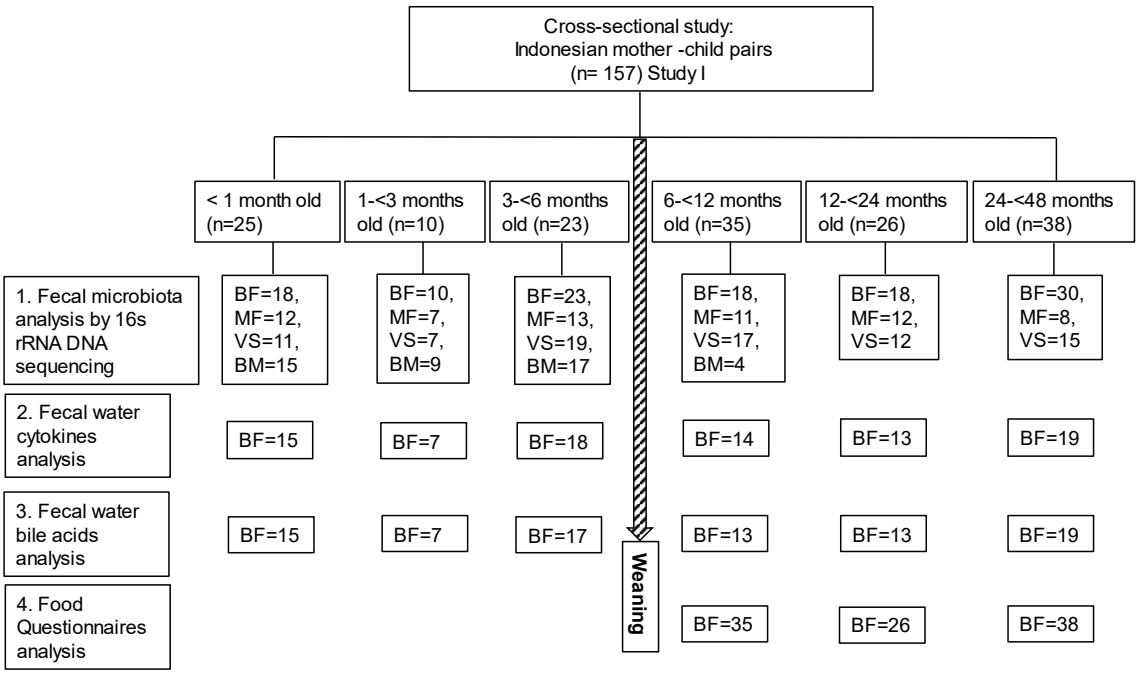


Figure 3. Flow chart of the study design and analyses of Study I. MF= Faecal sample of mother, VS= Vaginal swab sample of mother, BM= Breast milk sample of mother, BF= Faecal sample of children, n= number of samples.

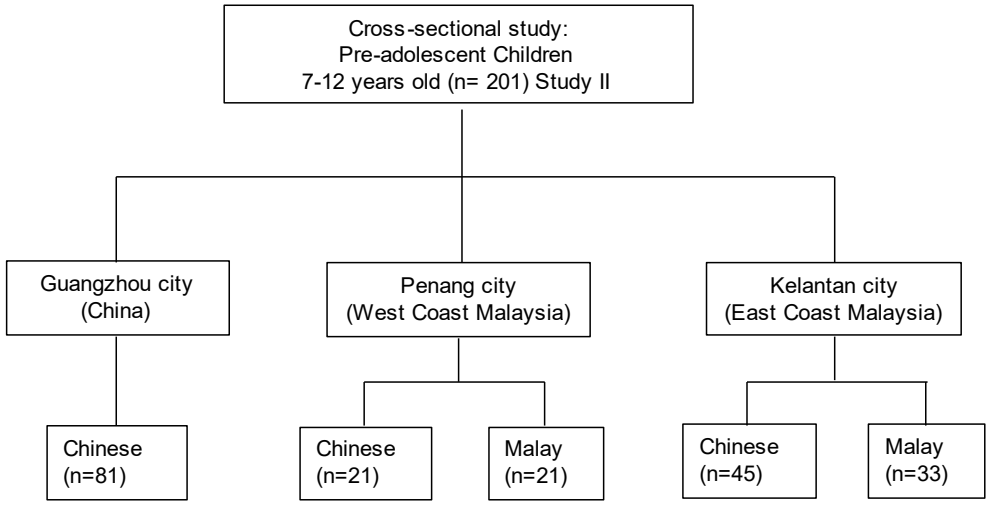


Figure 4. Flow chart of the study design for Study II. Faecal samples were collected for faecal microbiota analysis by 16s rRNA amplicon sequencing. Food questionnaires were analysed for food frequency consumption. N= number of samples.

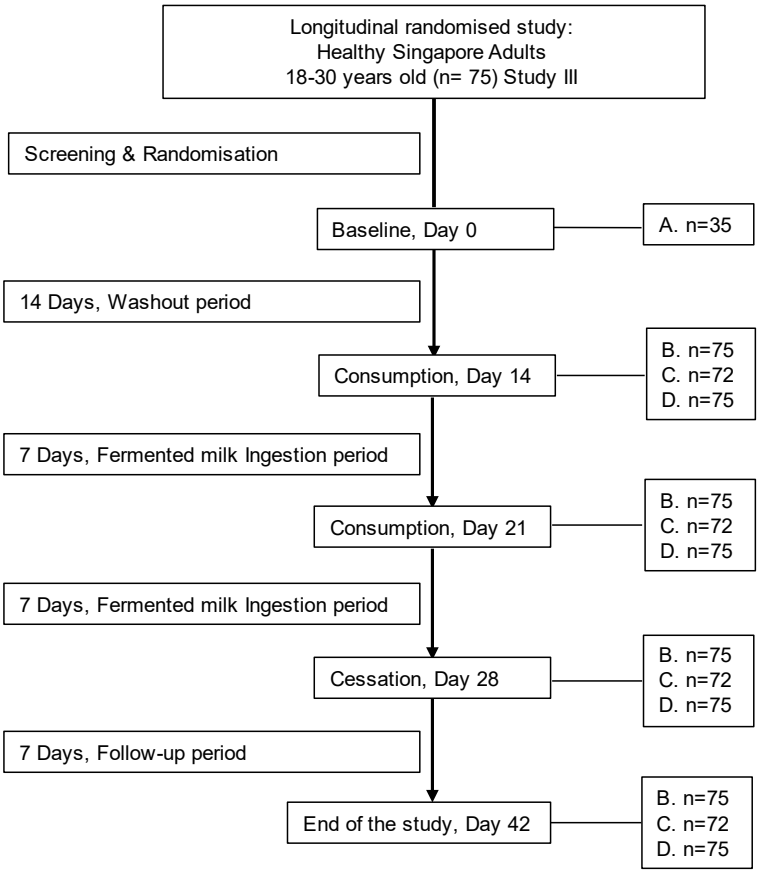


Figure 5. Flow chart of the study design and analyses of Study III. Faecal samples were collected. A= food questionnaire analysis, B= faecal microbiota analysis by 16s rRNA amplicon sequencing, C= faecal water cytokines analysis, D= Bristol stool characteristics, n= number of samples.

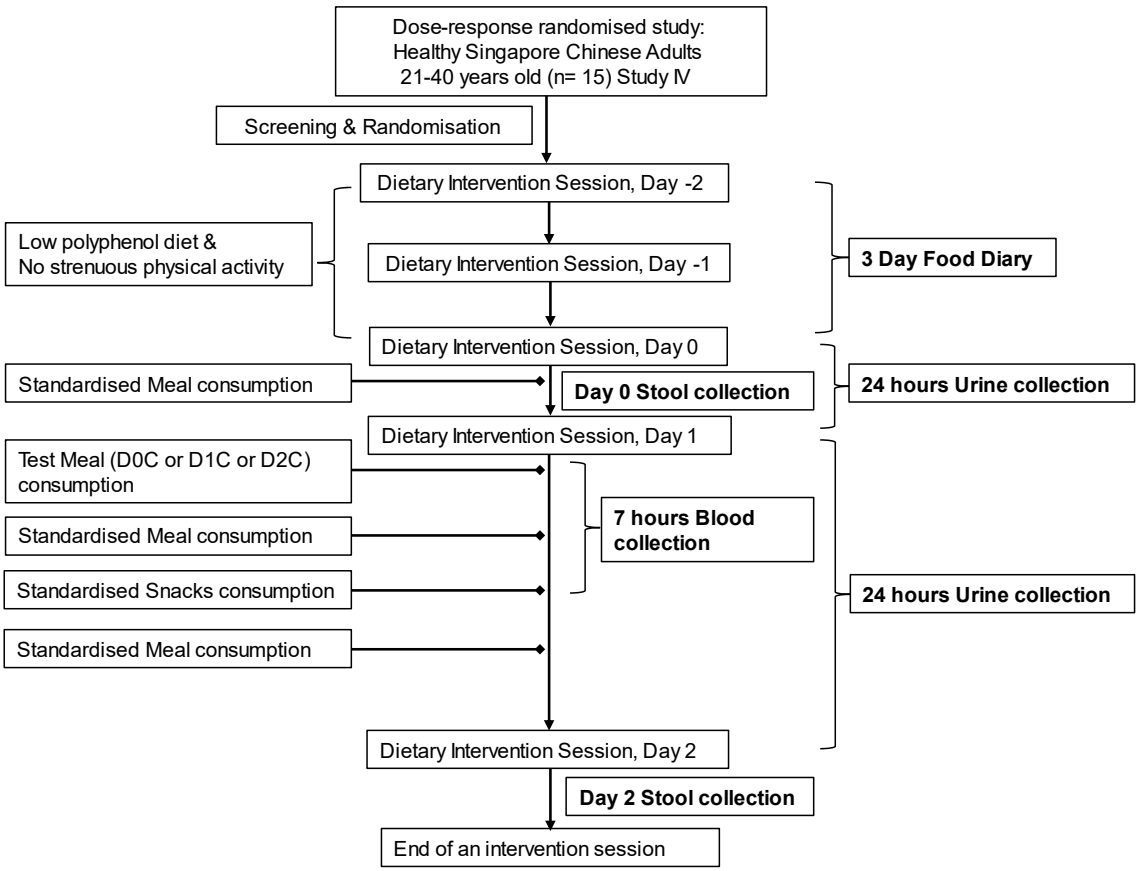


Figure 6. Flow chart of the study design and analyses of Study IV. Stool, 7-hour blood, and 24-hour urine collections were collected for faecal microbiota analysis by 16s rRNA amplicon sequencing, plasma phenolic acids analysis, and urine polyphenol analysis.

4.2 Methods

4.2.1 Collection of samples

Stool, vaginal swabs, breast milk, urine, and blood samples were collected. Study I obtained faecal samples from the mother and her child, lower vaginal swab, and breast milk from the mother at a one-time sample collection (Fig. 3). Study II collected faecal samples at a one-time sample collection (Fig. 4). Study III collected faecal samples at four-time points: baseline; 14 days after a washout period, seven days after consumption, 14 days after consumption, and 14 days after cessation of consumption (Fig. 5). In Study IV (Fig. 6), faecal samples were collected on Day 0: 3 days after washout, and Day 2: 1 day after the test meal consumption. Twenty-four hours of urine was collected twice on Day 0 to Day 1 (until the consumption of the

test meal) and Day 1 to Day 2 (24 hours after consumption of the test meal). A total of 13 blood samples at baseline (0 hour) and then at regular intervals were collected within 7 hours after consuming the test meal on Day 1. After being collected, the samples were transferred to the National University of Singapore laboratory with an appropriate cold chain.

4.2.1.1 Collection method for the faecal samples (Study I, II, III, IV)

The faecal samples were collected for the faecal microbiome analysis using 16s rRNA amplicon sequencing (Study I-IV), faecal water cytokines (Study I, III), and bile acids (Study I) analyses. Each participant was provided with a stool sample collection kit beforehand. The participants collected the 5- 10 g of faeces (3-5 scoops of the provided spatula without contaminating with urine or surroundings) delivered in a collection tube containing 2 ml of RNAlater® (Ambion Inc., Austin, TX, USA) preservative buffer. The weight of faeces was recorded and calculated. The samples were transferred on ice to the collection point on the appointed collection day.

4.2.1.2 Collection method for the vaginal samples (Study I)

The vaginal samples were collected for vaginal microbiome analysis using 16s rRNA amplicon sequencing. Nurses or medical doctors helped to swab the vaginal fluid from the lower part of vagina with a sterile cotton swab suspended in a collection tube containing 1 ml of Amies Transport solution. The samples were transported on ice and kept at -80º C until analysis.

4.2.1.3 Collection method for the breast milk samples (Study I)

The breast milk samples were collected for breast milk microbiome analysis using 16s rRNA amplicon sequencing. The mother collected approximately 10 ml of breast milk in the provided sterile container after cleaning the nipple and areola of the breast with a wipe of sterile saline solution. The transportation of the samples was on ice and stored at -80º C.

4.2.1.4 Collection method for the blood samples (Study IV)

The research nurses withdrew Venous blood into a 6 ml K_2EDTA tube (BD, Franklin Lakes, NJ, USA). The plasma samples were centrifuged at 1500 x g for 10 minutes at 4º C and stored at -80º C.

4.2.1.5 Collection method for the urine (Study IV)

Two 24-hour urine samples were collected separately in 3 L urine containers (Simport, Canada) on Day 0 and Day 1.

4.2.2 Collection of the demographic and anthropometric data

Basic demographic data such as age, sex, race, ethnicity, body weight, height, and concomitant medications were recorded for all studies. The stool characteristic was also recorded every day on Day 8-14 (baseline), Day 15-21 (first half of ingestion), Day 22-28 (second half of ingestion), Day 29-35 (first half of follow-up), and Day 36-42 (second half of follow-up) in Study III.

4.2.3 Collection of dietary intake questionnaires or records

Dietary intake questionnaires were collected at baseline or after consent or at the time of sample collection. The questionnaires in Study I (Appendix text 1) included the type of foods, frequencies of consumption, and portion size of the food items, while Study II (Appendix text 2) and Study III (Appendix text 3) contained data on the type of foods and food frequency. Study IV used the three-day food diaries during the run-in period of three days.

4.3 Sample processing

4.3.1 Faecal DNA extraction (Study I, II, III, IV)

Except for Study II, DNA was extracted for the faecal samples from all the studies using sets of chemicals. According to the weight of the collected sample, the faecal sample was dissolved with RNAlater® in 1:9. After that, 0.2 ml of faecal homogenate was washed twice with 1 x phosphate-buffered saline (PBS). The mixture of the faecal pellet was treated with 300 ml of tris-SDS and 500 ml of TE-saturated phenol (Sigma-Aldrich Cor., St.Louis, Missouri, USA) along with the 0.3 g glass beads (0.1 mm diameter) mechanical blasting by BeadBlaster 24 (Benchmark Scientific, Edison, USA). The lysate was centrifuged, and the supernatant was beads-blasted with 0.4 ml of phenol/chloroform/isoamyl alcohol (25:24:1) (Sigma-Aldrich Cor., St.Louis, Missouri, USA). DNA was precipitated with 250 ml of supernatant, 25 ml of 3 M pre-chilled sodium acetate (pH 5.2), and 0.3 ml isopropanol (Sigma-Aldrich Cor., St.Louis, Missouri, USA). The DNA was washed with 500 ml of 70% ethanol and dried at 60ºC for 30 minutes. The DNA was eluted in 200 ml TE buffer (pH 8.0).

For Study II, faecal DNA was extracted using TIANamp Stool DNA kit (Tiangen Biotech, Co., Ltd., Beijing, China) according to the manufacturer's protocol.

4.3.2 Vaginal swab sample DNA extraction (Study I)

After resuspending, 0.5 ml of aliquot was lysed by 0.05 ml of lysozyme, 35 ml of mutanolysin and 1 ml of lysostaphin, and 50 ml of TE50 buffer (pH 8.0) (Sigma-Aldrich Cor., St.Louis, Missouri, USA) for 1 hour at 37ºC. The mixture was beads-blasted by 0.05 g of glass beads (0.1 mm diameter) and BeadBlaster 24 (Benchmark Scientific, Edison, USA). The following DNA extraction was performed using the QIAamp DNA Mini kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's protocol.

4.3.3 Breast milk DNA extraction (Study I)

The fat layer of the five ml breast milk sample was separated by centrifugation, and the supernatant was discarded. Once the pellet was washed, it was mixed with 0.5 ml of proteinase K (Qiagen GmbH, Hilden, Germany), 0.08 ml of RNAase A (Sigma-Aldrich Cor., St.Louis, Missouri, USA), and 0.2 ml of buffer AL (Qiagen GmbH, Hilden, Germany). It was incubated at 56ºC for 10 minutes and washed with ethanol. The following DNA extraction was conducted using the QIAamp DNA Mini kit (Qiagen GmbH, Hilden, Germany) and following the manufacturer's protocol.

4.3.4 16s rRNA amplicon sequencing (Study I, II, III, IV)

Once the extracted DNA was quantified, each sample was normalised to 12.5 ng to proceed for amplification with Nextera® transposase sequences at v3 and v4 regions of the 16s rRNA gene and KAPAHiFi™ HotStart ReadyMix kit (Roche life science, Inc., Wilmington, MA, USA). Nextera XT indices and adapter sequences (Illumina, Inc., San Diego, CA, USA) were added to the amplified polymerase chain reaction (PCR) products after being purified with Agencourt® AMPure XP beads (Beckman Coulter, Inc., Fullerton, CA, USA). The purified library was quantified with Quanti-iTTM PicoGreen® dsDNA kit (Invitrogen, Inc., Carlsbad, FA, USA) and qualified with Agilent high sensitivity DNA kit (Agilent Technologies, Inc., Santa Clara, CA, USA). All the libraries were normalised to the optimized concentration, and then they were pooled. The pooled amplicon library (PAL) was quantified using KAPA library quantification kit (Roche life science, Inc., Wilmington, MA, USA) in ABI 7500 real-time PCR system (ThermoFisher Scientific, Inc., Waltham, MA, USA). PAL was denatured and diluted until the required titrated concentration. The

denatured amplicon library was spiked with the PhiX control library (Illumina, Inc., San Diego, CA, USA) and sequenced in the Miseq sequencing system (Illumina, Inc., San Diego, CA, USA).

4.3.5 Faecal water preparation (Study I and III)

One gram of collected faecal sample was treated with a mixture of 0.01 M Phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich Cor., St.Louis, Missouri, USA) and 1% Bovine Serum Albumin (BSA) in PBS solution. After centrifuging, all the supernatants were stored at -80ºC for analysis of faecal water cytokines or bile acids.

4.3.6 Faecal water cytokines analysis (Study I and III)

The concentration of 11 cytokines: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IFN-ɣ, TNF-α, and GM-CSF were measured in the extracted faecal water using the LUNARIS™ Human 11-Plex cytokine kit (AYOXXA Biosystems GmbH, Köln, Germany) according to the protocol of kit. The LUNARIS™ BioChip included the prepared diluted samples, standards, and blanks which were read with a fluorescence microscope (Zeiss Axio Imager M2, Zeiss, Germany). Fluorescence quantification was calculated using the LUNARIS™ analysis suite in the LUNARIS™ accessory kit (AYOXXA Biosystems GmbH, Köln, Germany). The concentration was reported in pg/ml.

4.3.7 Faecal water bile acids analysis (Study I)

In Study I, the following bile acids were identified and measured: chenodeoxycholic acid (CDCA), colic acid (CA); ursodeoxycholic acid (UDCA), deoxycholic acid (DCA), lithocholic acid (LCA); glycochenodeoxycholic acid (GCDCA), taurochenodeoxycholic acid (TCDCA), taurocholic acid (TCA), glycocholic acid (GCA), glycodeoxycholic acid (GDCA), glycolithocholic acid (GLCA), hyodeoxycholic acid (HDCA), tauroursodeoxycholic acid (TUDCA), glycoursodeoxycholic acid (GUDCA), taurodeoxycholic acid (TDCA), alpha- and beta-tauromuricholic acid (TMCA [a + b]) taurolithocholic acid (TLCA). Supernatant from the faecal water was extracted using the faeces extraction buffer (Sigma-Aldrich Cor., St.Louis, Missouri, USA). After shaking, sonication, and centrifugation, bile acids from the extracted faecal water were measured using Biocrates® bile acids kit (Biocrates life sciences, AG, Austria), following the manufacturer's protocol. The Agilent 1290 infinity high-performance liquid chromatography system (Agilent Technologies Inc., Santa Clara, CA, USA) coupled

to the AB SCIEX QTrap 5500 mass spectrometry (AB SCIEX Pte. Ltd., Framingham, MA, USA) detected the bile acids. The concentration of bile acids was computed using MultiQuant 3.0 software SCIEX (AB SCIEX Pte. Ltd., Framingham, MA, USA). It was expressed as umol/g in the wet weight of faeces.

4.3.8 Plasma Phenolic acids analysis (Study IV)

Many phenolic acids were measured and described in detail in Haldar's paper (Haldar et al., 2018). The data on Cinnamic acid (CA) and phenylacetic acid (PAA) were used in this study to correlate with the change in the gut microbiome because the previous paper (Haldar et al., 2018) reported that they were significantly changed across the different doses of spices. Both plasma phenolic acids were extracted using the protein precipitation method, and reconstitution was performed. An Agilent Infinity 1290 Liquid Chromatography system (Agilent Technologies Inc., Santa Clara, CA, USA) coupled to a SCIEX Triple Quad 3500 mass spectrometry (SCIEX Pte. Ltd., Vaughan, ON, Canada) detected plasma phenolic acids. MultiQuant 3.0 software SCIEX reported the data.

4.3.9 Polyphenols analysis of the urine (Study IV)

Urinary total polyphenol excretion (TPE) was analysed with the solid phase extraction and Folin-Ciocalteu assay (Sigma-Aldrich Cor., St.Louis, Missouri, USA) (Medina-Remón et al., 2009). Urinary TPE was calculated from the measured absorbance and described as mg gallic acid equivalent (GAE) per mmol creatinine. The detailed procedure was described in the previously published paper (Haldar et al., 2019).

4.3.10 Stool characteristics (Study IV)

A Bristol stool form scale: types 1-6 (Lewis and Heaton, 1997) was used to determine the stool characteristics, and the mean value of scores per week was described. The frequency of defecation was recorded and reported as the frequency of defecation per week.

4.4 Data analysis

Data analysis and visualisation were performed in Quantitative Insights Into Microbial Ecology (QIIME) version 1.9.1 (Caporaso et al., 2010), GraphPad Prism 7 and 8 software (GraphPad, Inc., San Diego, CA, USA), Canoco5 software

(Microcomputer Power, Ithaca, NY, USA) and R Studio software (RStudio, Inc., Boston, MA, USA).

4.4.1 Bioinformatics analysis of the 16s rRNA amplicon sequencing outputs (Study I, II, III, IV)

The output data from Miseq sequencer were analysed with QIIME version 1.9.1 (Caporaso et al., 2010). The individual sample's forward and reverse reads were joined, and the demultiplexed paired reads were filtered for a quality Q-score of 25. Non-chimera sequences were selected after the chimera were filtered and excluded using USEARCH v6.1 (Edgar. 2010). Operational taxonomic units (OTU) were chosen using the open-reference OTU picking method and Greengenes v13_8 reference database at 97% similarity. OTUs were mapped to profile the taxonomical level up to the bacterial genera. All studies followed the above workflow to obtain the abundances of the relative proportion of the bacterial taxonomy levels. The relative abundances of bacterial genus-level OTUs are presented on a principal component analysis (PCA) plot (Study I) and a distance-based redundancy analysis (db-RDA) plot (Study II and III) based on the square-root of the Bray Curtis distances. The biplots were drawn by the CANOCO 5 software package (Microcomputer Power Co, Ithaca, NY, USA). Alpha diversity indices of species were computed using the Greengenes v13_8 database, and Chao 1 and Shannon indices were presented in Study III and IV. Beta diversity was generated from the weighted and unweighted UniFrac distance matrices by a Qiime script (Study II) and R package (Study III) (McMurdie and Holmes 2013). They were visualised using principal coordinate analysis plots (PCoA) by a Qiime script (Study II) and R v4 (Study III).

4.4.2 Clustering analysis for enterotyping (Study II and III)

Applying the enterotyping method (Arumugam et al., 2011 and Costea et al., 2018), the relative abundance of bacterial genera of all the data (Study II) and Timepoint 2 data (Study III) were calculated using the Jensen-Shannon distance (JSD) matrix. Based on JSD, clustering was performed with the partitioning around medoids (PAM) algorithm. The Calinski-Harabasz (CH) index estimated the optimal number of clusters and validated it by the individual and average silhouette coefficient (Si). PCoA plot using the JSD matrix and adegraphics R package was visualised for Study II.

4.4.3 Correlation index (Study I)

A correlation index was used to measure the degree of correlation between the commensal gut bacterial species and well-known potential pathogens in Study I. It is the summation of correlation coefficient values (r) of those bacteria in a group of infants. In Study I, the correlation of bacteria found in the groups of infants before weaning and after weaning were compared and correlation indexes were applied. The 15 commensal species included were *Akkermansia*, *Bifidobacterium*, *Blautia*, *Enterococci*, *Faecalibacteria*, *Lactobacilli*, *Ruminococci*, *Escherichia coli*, other *Bacilli*, other *Bacteroides*, other *Campylobacter*, other *Clostridia*, other *Prevotella*, other *Staphylococci*, and other *Streptococci*. 14 well-known potential pathogens included were *Neisseria*, *Pseudomonas*, *Bacteroides fragilis*, *Campylobacter ureolyticus*, *Clostridium difficile*, *Clostridium perfringens*, *Haemophilus influenzae*, *Haemophilus parainfluenzae*, *Klebsiella*, *Prevotella melaninogenica*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus agalactiae*, *and Streptococcus anginosus*. A zero-value correlation index meant that there was no net correlation between the commensal species and pathogens. In Study I, the maximum positive correlation index was 168 (15 commensals x 14 pathogens x 0.8), whereas the greatest negative correlation was -84 (15 commensals x 14 pathogens x [-0.4]).

4.4.4 Analysis of dietary intake (Study I, II, III and IV)

Dietary analyses were performed based on the questionnaire type (Appendix texts 1, 2, and 3). Study I included the portion size of the food item; therefore, it reported the total amount of daily consumption in grams. The questionnaires used in Study II analysed the frequency of food consumption over a month, however, the food item's portion size was not considered a reliable answer. The same method was applied to the analysis of the food questionnaire in Study III, but the frequency of food consumption per week was calculated.

A food diary record was used in Study IV for three days during the run-in period before the test meal was consumed. The food item listed in the diary was converted to energy (kcal) and nutrients such as carbohydrate, protein, total fat, saturated fat, fibre, cholesterol, and sodium following the online Energy and Nutrient Composition of Food tool developed by the Singapore health promotion board (Energy & Nutrient Composition of Food, Health Promotion Board, Singapore). The portion of the food item was regarded in the calculation which means that the daily intake of energy and nutrients were reported.

The food items were categorised into 10, 15, and 6 types for Study I, II, and III, and the details are described in Appendix texts 1, 2, and 3. Study I recorded ten food types such as complex carbohydrates, vegetables, legumes and soy, fruits, meats, dairy products, snacks, beverages, fast foods, and seasonings, where each food

item's frequency and serving size were calculated. The frequency of consumption was counted per day. The amount of consumption of food items under the same food type was combined and converted into grams. Study II reported 15 food types: complex carbohydrates enriched foods, oily foods, vegetables, refined sugars enriched foods, dairy products, fruits, meats, protein-enriched plants, eggs, seafood, caffeinated drinks, biscuits/pastries/cakes, Yoghurt, preserved foods, and curry. It described the frequency of monthly food consumption. Study III categorised food types into carbohydrate-rich foods, protein-rich foods, vegetables, fruits, nuts, and beverages. The total frequency of food items was reported during the week. All the participants completed their dietary intake questionnaires or records for Study I, II, and IV but only 35 food frequency questionnaire answers were analysed for Study III due to the quality of responses and missing data.

4.4.5 Statistical analysis (Study I, II, III, IV)

The statistical analysis in all the studies was performed using GraphPad Prism 7 and 8 software (GraphPad, Inc., San Diego, CA, USA), QIIME (Caporaso et al., 2010), R 3.5.1, 3.5.2, and 4 software (R Studio, Inc., Boston, MA, USA).

A permutational multivariate analysis of variance (PERMANOVA) test was done, with the Bray Curtis or JSD distances followed by a Bonferroni multiple pairwise comparison test with a Monte Carlo permutation test at 4999, to compare the dis-similarities of the bacterial genera communities using the pairwiseAdonis R package (Martinez AP 2019). An analysis of similarities (ANOSIM) was used, with the beta diversity Unifrac distances to compare dis-similarities of diversities between the groups using QIIME (Study II).

All the data were checked for normality in nature and proceeded with the appropriate statistical methods for further analysis. Mean, and Standard Deviations (SD) were calculated for daily energy intake and nutrients (Study IV). Matched data intra-individual variation at baseline between the interventions was presented as a mean percent coefficient of variation (%CV) (Study IV). A parametric one-way analysis of variance (ANOVA) and Bonferroni pairwise comparison tests or mixed-effects analysis tests were applied for alpha diversity analysis in Study III or daily intake of energy and nutrients in Study IV. The data of bacterial genera abundances (Study I), cytokines (Study I and III), and bile acids (Study I) between the groups were analysed by two-way analysis of variance followed by Bonferroni multiple comparison tests or mixed-effects model.

To compare the relative abundance data of bacterial genus and dietary intake between the groups, a non-parametric Mann-Whitney U test (Study II and III) and Kruskal-Wallis test followed by post-hoc multiple comparison Dunn's test (Study I and II) were performed. To compare the bacterial abundances (Study III, IV) and

alpha diversity changes (Study IV) for different time points or intervention groups, a non-parametric matched Friedman and Nemenyi post-hoc multiple pairwise comparison tests were used. The correlation between abundances of the bacterial genus (Study I, II, IV) dietary intake amount (Study I, II) potential pathogens (Study I) and changes in the three metabolites (Study IV) were checked by a non-parametric Spearman test. The simple linear regression test was applied to the relation between the absolute abundance of two bacteria at baseline and their changes (study IV). The significance of the results was set to a p-value < 0.05.

4.5 Ethical approval and consent

All studies were undertaken in line with the guidelines of the declaration of Helsinki and the respective country's Good Clinical Practice. The study protocols and research-related documents were approved by the individual research review bodies such as the Domain Specific Research Board (DSRB); Singapore, Institutional Review Board (IRB) at National University of Singapore, Gadjah Mada; Yogyakarta, Guangzhou, Penang, Kelantan Universities. Before initiating the studies, informed written consents were obtained from all the volunteers (Study I, III, IV) and mothers or guardians on behalf of the children (Study I and II).

5 Results

The top bacterial genera found in this thesis across the different ages from neonates to adults in the studies, are summarised in figure 7. The figure highlights the dominant bacterial genera across the different ages of neonates (less than one-month-old), children (7-12 years old), and adults (up to 40 years old) in the studies involved. Study I was divided into six age groups for the babies, and six genera (*Prevotella*, *Bacteroides*, *Lactobacillus*, *Peptostreptococcus*, *Staphylococcus*, *Streptococcus*, *Bifidobacterium*) were prevalent in the different types of samples (faecal, vaginal swabs, breast milk) from the Indonesian mothers and babies. The second study (II) collected faecal samples from children (7-12 years old), and *Bacteroides* and *Prevotella* were dominant according to their bacterial clusters compared to their ethnicities and living locations. In Study III, the three clusters (*Bacteroides*, *Prevotella*, *Blautia*) modulated their basal faecal genera after *Lactobacillus* administration (Timepoint 1 to 4). The last study (IV) with an intervention of three doses of spices showed the abundances of *Bacteroides* were the highest before and after the spice consumption in changes of *Bifidobacterium*.

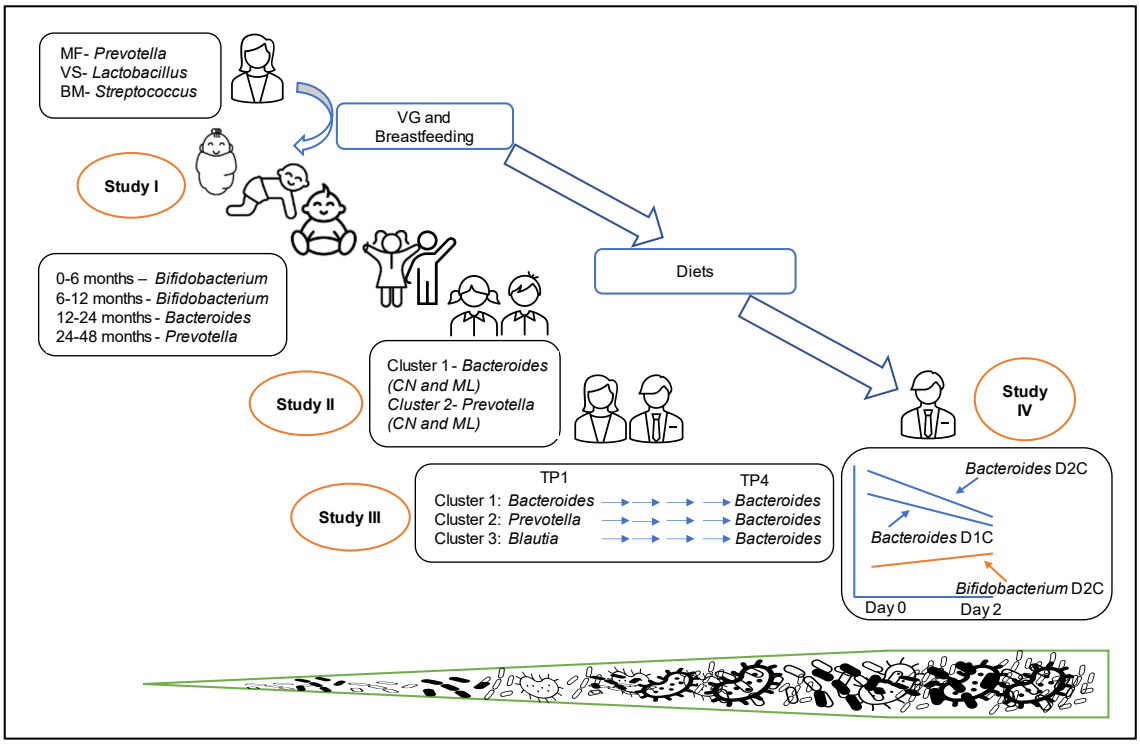


Figure 7. Overview of the top bacterial genera found across the different ages from the neonates (less than one-month-old) to the adults (40 years old) in the four studies. The top bacteria may or may not be statistically significant within the group. MF= faecal samples of mothers, VS= vaginal swabs samples of mothers, BM= breast milk samples of mothers, BF= faecal samples of children, D1C= Meal with low (6 g) mixed spices intervention, D2C= Meal with high (12g) mixed spices intervention, CN= Southern Chinese, ML= Malay, VG= Vaginal delivery, TP= Timepoint. The bacteria shown were the top genera among the faecal microbiota of the group.

5.1 Composition of faecal microbiota in the children (Study I and II)

5.1.1 Transmission of microbiota from mothers to children (Study I)

We determined the microbiota of mother and child pairs who had been classified into six time-line groups, neonates (from birth to one-month-old), infants (1-3-months, 3-6-months, 6-12-months), toddlers (12-24-months), and pre-schoolers (24-48-months). The 6-12 months period was the weaning time point.

5.1.1.1 Comparison of faecal microbiota profiles of children and their mothers (highlight for aim no. 1. a)

The faecal microbiota profile of infants varied from their maternal faecal microbiota (Fig. 8a). The profile of the 12-24-month age group started to shift towards the profile of the mothers, and the 24-48-month group coincided with the maternal faecal microbiota. The older the children's age, the more the mothers and children shared faecal microbiota, especially after weaning (6-12 months). The faecal microbiota of the children shared from 5.2% to 34.3% of that of the mothers across the different age groups (Table 1). Some faecal bacteria in children were similar to their mothers in some age groups, while some were significantly different from their mothers' bacteria (Fig. 9a). More specifically, in the neonate group, the abundance of *Bifidobacterium* was the highest in infants, but, *Prevotella* was the most prevalent in their mother's faeces throughout the age groups, except at 1-3 months, during which it was *Bacteroides*. The abundance of maternal *Bifidobacterium* was 6-0.9% (2.5% mean ± 4.5% SD) across the age groups. The relative abundances of *Bifidobacterium*, *Bacteroides*, an unknown genus of *Enterobacteriaceae,* and *Klebsiella* were reduced with the increasing age of the infants. *Bifidobacterium* and *Bacteroides* were the first and second most abundant genera from the neonatal period to 12-24 months. Both bacteria were significantly different from the mother's *Bifidobacterium* and *Bacteroides* abundances until 24 months. The abundances of *Enterobacteriaceae* and *Klebsiella* reached below 1% of total OTUs at 24-48 months and 12-24 months, and their quantities were comparable with their mothers at 6-12 months and 1-3 months onwards, respectively. The abundances of some taxa were very low before three months of age but populated gradually after that, such as *Prevotella* and *Lachnospiraceae* (started to increase from three months onwards) and *Faecalibacterium*, *Blautia,* and *Ruminococcaceae* (six months onwards). *Prevotella* became more than 10% of the total faecal OTUs in most children (66.7%) in the 24-48 month group. The abundances of these bacteria (except *Ruminococcaceae*) reached similar abundances of their mothers at 12-24 months.

Table 1. Shared OTUs between the mothers' samples and the faecal samples of the children (%). (Study I)

Category	Percentage of shared OTUs in the samples of mothers with the faecal samples of children (%)		
	among the faecal samples of mothers	among the vaginal swabs of mothers	among the breast milk samples of mothers
< 1-month-old age group	5.2	9.2	11
1- < 3 months old age group	5.8	12.3	9.3
3- < 6 months old age group	6.6	10.1	10.1
6- < 12 months old age group	10.6	15	11.1
12- < 24 months old age group	18.5	8.3	-
24- 48 months old age group	34.3	19.1	-

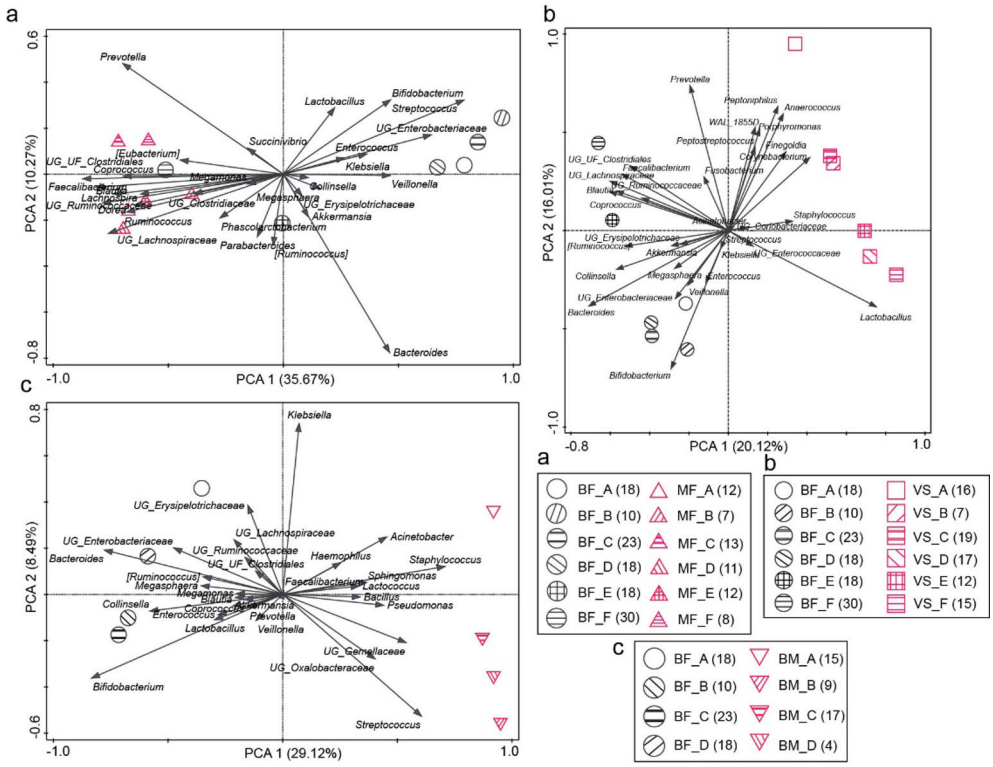


Figure 8. Biplots of the principal component analysis (PCA) of (a) the faecal microbiota of children and their mothers (b) the faecal microbiota of children and vaginal microbiota of their mothers (c) the faecal microbiota of children and breast milk microbiota of their mothers using the square root Bray-Curtis distance matrix of the relative abundances of bacterial genera. Number of samples is indicated in parentheses. BF= faecal samples of children, MF= faecal samples of mothers, VS= vaginal swabs samples of mothers, BM= breast milk samples of mothers, A-F= less than 1 month, 1-3 months, 3-6 months, 6-12 months, 12-24 months, and 24-48 months age groups, respectively. (Study I)

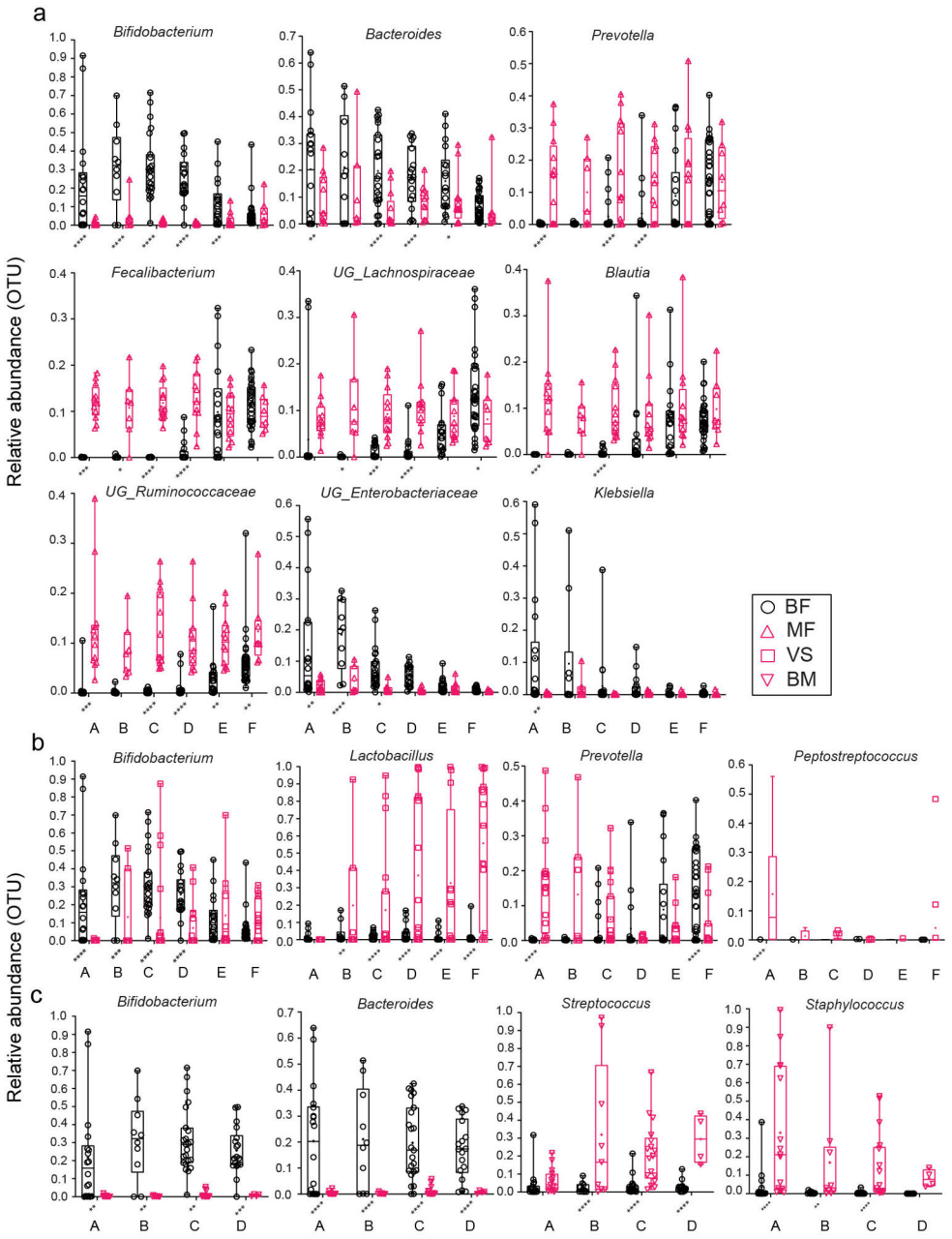


Figure 9. The relative abundances of the significant bacterial genera among 1% and above of total OTUs comparing (a) the faecal microbiota of children and their mothers (b) the faecal microbiota of children and vaginal microbiota of their mothers (c) the faecal microbiota of children and breast milk microbiota of their mothers. * $p \leq 0.05$ comparison between the pair. BF= faecal samples of children, MF= faecal samples of mothers, VS= vaginal swabs samples of mothers, BM= breast milk samples of mothers, A-F= less than 1 month, 1-3 months, 3-6 months, 6-12 months, 12-24 months, and 24-48 months old age groups, respectively. The complete figures can be found in Paper I. (Study I)

5.1.1.2 Comparison of the faecal microbiota profiles of children and the vaginal microbiota of their mothers (highlight for aim no. 1. a)

The faecal microbiota profiles of the children were clustered separately from the maternal vaginal microbiota throughout the age groups (Fig. 8b). The shared faecal microbiota of neonates (less than one month) was 9.2% among vaginal microbiota (Table 1). *Lactobacillus* had the highest abundance among the vaginal microbiota across the age groups, apart from the first month of life, in which *Peptostreptococcus* dominated (Fig. 9b). The abundance of maternal vaginal *Prevotella* decreased along with the age time points, while that of the children increased after 24 months. The opposite trend was seen for *Bifidobacterium* in the maternal vaginal microbiota and the faecal microbiota of children.

5.1.1.3 Comparison of the faecal microbiota profiles of infants (up to 1-year-old) and the breast milk microbiota of their mothers (highlight for aim no. 1. a)

The faecal microbiota profile of infants was distinct from the maternal breast milk microbiota profile (Fig. 8c). The faecal microbiota of the neonates shared 11% of the breast milk microbiota (Table 1). *Staphylococcus* was the dominant taxon among breast milk microbiota during the neonatal period (less than one month), and subsequently, *Streptococcus* was the most abundant genus. The abundances of *Bifidobacterium*, *Prevotella*, *Bacteroides,* and *Lactobacillus* were lower than 1% of the total OTUs among the breast milk microbiota in all the age groups; however, *Bacteroides* and *Lactobacillus* reached 1% in the 3-6 months age group. *Lactobacillus* in the infants' faecal microbiota were detected in 1-4% of the total OTUs among faecal microbiota (this data is not shown in Fig. 9c).

5.1.2 Correlation of commensal gut species and potential pathogens in children (Study I)

Commensal gut species and potential pathogens were examined for their correlation before (< 6 months) and after weaning (6-48 months) periods (Appendix Fig. 1). The correlation index was applied for each period. This allows an understanding of the correlation between commensals and potential pathogens after the diet changes. Positive and negative correlations suggest that the two groups of species associate together or have the same trend and behave in an opposite manner or have the opposite trend. Zero correlation means the two groups have no association. Many negative correlations (46%) were found before weaning, and the correlation index was 4.85. *Bifidobacterium* and *Klebsiella* species were statistically associated as opposites, whereas two other species pairs were positively associated before

weaning. Few negative correlations (18%) were observed during the weaning period and onward, and its correlation index was 30.97. All 12 species pairs were positively correlated, and *Hemophilus parainfluenzae* was associated with five commensal species.

5.1.3 Faecal microbiota of pre-adolescent children (Study II)

This study compared the faecal microbiota of (1) two ethnicities dwelling in the same city and (2) the same ethnicity dwelling in different cities: Southern Chinese and Malay in Guangzhou, Penang, and Kelantan. The overview of the findings of the statistical analysis tests is summarised in Table 2.

Table 2. Overview of the results of the Statistical analysis tests performed in Study II comparing the faecal microbiota of two clusters between two ethnicities in the same city or between two cities of the same ethnicity

Pairs	Unweighted unifrac β diversity	Weighted unifrac β diversity	Clustering in JSD distance		Clustering in B-C distance		Abundances of major bacterial genera	
Cluster			1	2	1	2	1	2
GZ	-	-	x		*		*n=2	
KL_CN	-	-	x		*		*n=4	
KL_ML	-	-	x		*		*n=1	
PN_CN	-	-	x		x		-	
PN_ML	-	-	x		*		*n=1	
KL_CN VS. KL_ML	*	x	x	x	x	x	x	x
PN_CN VS. PN_ML	x	x	x	x	x	x	x	x
GZ VS. KL_CN	*	*	x	x	*	x	*n=5	*n=4
GZ VS. PN_CN	*	x	x	x	*	x	*n=4	x
KL_CN VS. PN_CN	*	x	x	x	x	x	x	x
KL_ML VS. PN_ML	*	*	x	x	*	x	*n=2	*n=2

KL= Kelantan city, PN= Penang city, GZ= Guangzhou city, CN= Southern Chinese, ML= Malay, JSD= Jensen-Shannon distance, B-C= Bray-Curtis distance, *= Significant differences between pairs * p≤ 0.05, x= No significant differences between pairs, n= The number of genera which was significantly different between the compared pair.

5.1.3.1 Effect of ethnicity and geographic location on faecal microbiota (highlight for aim no. 2)

The faecal samples were clustered into two optimised clusters using a Partitioning Around Medoids (PAM) method, the Jesen-Shannon distance (JSD), and 175 identifiable genera relative abundances (Appendix Fig. 2). This was done in order to investigate the effect of ethnicity or geographic location of cities as causative factors on children's faecal microbiota. Of the children 68.2% and 31.8% were classified into Cluster 1 and 2, respectively. The faecal microbiota abundances when comparing the two clusters of Chinese from Guangzhou and Kelantan, and the Malay from Kelantan and Penang were varied but not for Penang Chinese comparison (first five rows, last column of Table 2). Clustering determines the classification of the types of bacteria.

The faecal microbiota when comparing Chinese and Malay populations from Penang and Kelantan, did not separate well in the beta diversity of the unweighted (types of bacteria presences) and weighted (quantity of bacteria presences) UniFrac distances (Appendix Fig. 3, Table 2), or the clustering by JSD or Bray-Curtis distances and abundances of major genera (Appendix Fig. 2 and 6th and 7th row last three columns of Table 2). However, the types of bacteria present in Kelantan Chinese slightly separated from those of the Malay population (sixth row, first column of Table 2) because the unweighted beta diversities resulted in significant differences ($p= 0.001$, $R= 0.164$) between the two ethnicities in the Kelantan children.

Differences in the diversity of faecal microbiota of the same ethnicity between two cities were seen in the unweighted Principal Coordinates Analysis (PCoA) plot (Appendix Fig. 4, last four rows, first column of Table 2), which shows the types of faecal microbiota of the Chinese or Malay living in any of the cities (GZ or KL or PN) were different. Similarly, the weighted beta diversity comparing Chinese from Guangzhou vs. Kelantan and Malay from Kelantan vs. Penang varied (Appendix Fig. 4 and Table 2). On the other hand, the diversities of the faecal microbiota in Chinese from Guangzhou vs. Penang and Kelantan vs. Penang were comparable. The faecal microbiota of the Chinese in Guangzhou and Kelantan or Penang from Cluster 1 had shared differences (last four rows of Table 2). The same trend occurred when comparing Malay children from Kelantan and Penang in Cluster 1 but not Chinese children from Kelantan and Penang.

The effect of ethnicity or geographic location factors on the faecal microbiota of children can be differentiated by analysing relative abundances of Operational Taxonomy Units (OTUs) above 1% of the total bacterial genera. Cluster 1 and 2 were dominated by 20 genera led by *Bacteroides* and seven genera led by *Prevotella* among 1% above OTUs. A total of 17 genera were seen as being significantly different between the two clusters using the Man-Whitney U test; among them, 14 were from Cluster 1, and three were from Cluster 2 (Appendix Fig. 5 a, b). The ratio

of the two bacteria was significantly different between the two clusters comparing the different ethnicities and cities but comparable within the same cluster (Appendix fig. 5 c). Interestingly, the relative abundance of *Bifidobacterium* in Cluster 1 and 2 from Guangzhou was less than 1% of total OTUs, while the other two cities had more than 1%.

When comparing the abundances of bacterial genera between two clusters of the same ethnicity, and city (apart from Penang Chinese), four bacterial genera: *Bacteroides*, *Prevotella*, *Parabacteroides*, *Streptococcus,* were observed to be significantly different (Appendix Table 1). These may contribute to the separation of clusters. Within the same city and cluster, the faecal microbiota of the different ethnicities was similar (Appendix Table 1). However, eleven bacterial genera showed differences in abundance between the different cities but with the same ethnicities (Appendix Table 1). It can be implied that the effect of the geographical location, but not the ethnicity factor, is the major contributing factor to bacterial abundance. Diet is associated with geographical location, which is one of the major causative factors in the faecal microbiota of children.

5.2 Faecal microbiota of adults (Study III and IV)

The effects of dietary intervention (fermented milk; Study III and dietary spices; Study IV) on the faecal microbiota of adults were studied in the 18-30 (Study III) and 21-40 (Study IV) year age groups.

5.2.1 Faecal microbiota composition at baseline

5.2.1.1 Clustering of basal faecal microbiota and microbial diversities before fermented milk intervention (Study III) (highlight for aim no. 3. a)

Three clusters: Cluster 1 (n=31), Cluster 2 (n= 14), and Cluster 3 (n=30), were classified according to 26 genera that present above 1% of total OTUs at baseline before the fermented milk consumption (Fig. 10, Appendix table 2). Cluster 1 had a prevalence of *Bacteroides* (30%), whereas its abundance at 8% and 12% in Cluster 2 and 3 were significantly different from the other two clusters. The abundances of *Sutterella* and *Megasphaera* were greater in Cluster 1 compared to the other two clusters, but an unknown genus of *Ruminococcaceae* (3% vs. Cluster 2; 6% and Cluster 3; 8%) was statistically lower. *Prevotella* (21%) was the top genus in Cluster 2, but it was low in Cluster 1 (0.2%) and Cluster 3 (1%) and significantly different. *Erysipelotrichaceae* (0.8%) and *Collinsella* (1.7%) were the highest in Cluster 2 and significantly different from Cluster 1 and 3. *Blautia* (16%) exhibited the highest

abundance in Cluster 3, while the other clusters had 12% each. *Ruminococcus* (4%) was found in a significant proportion in Cluster 3, and this was significantly different from other clusters. The proportion of each genus observed in Cluster 3 was relatively more similar than those in Cluster 1 and 2. Among the 26 genera, the relative abundances of 11 were comparatively higher in Cluster 3 than those of the other two clusters, whereas 9 and 6 bacteria were more abundant in Cluster 1 and 2. This suggested that Cluster 3 had no predominant genera, which was distributed evenly when compared with the other clusters. The lactic acid bacteria *Bifidobacterium* (Cluster 1; 4%, Cluster 2; 3% and Cluster 3; 5%) and *Lactobacillus* (Cluster 1; 0.3%, Cluster 2; 0.8% and Cluster 3; 0.6%) were comparable among the three clusters.

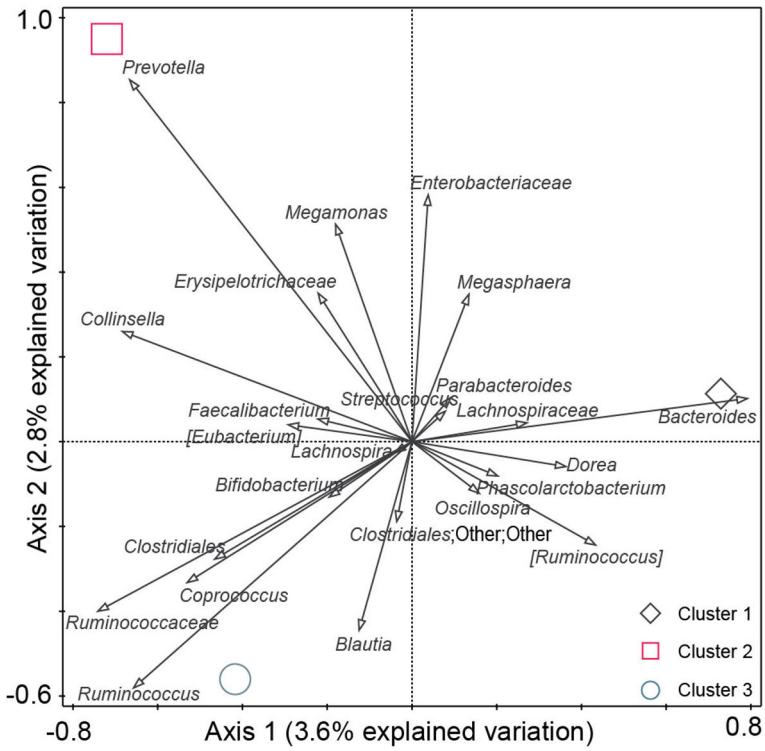


Figure 10. Biplot of db-RDA analysis using the square root the Bray-Curtis distance matrix based on the major faecal microbiota in the three clusters at baseline. (Study III)

The species richness and evenness of the alpha diversity in the indexes of Chao 1 and Shannon were comparable in all clusters at baseline (Appendix Fig. 6 a and b). The unweighted and weighted UniFrac distances of both types and the quantities of species of beta diversity were different between the clusters at baseline (Appendix Fig. 6 c and d).

5.2.1.2 Basal faecal microbiota and microbial diversities before an intervention with dietary spices (Study IV) (highlight for aim no. 4)

The relative abundances of the major bacterial genera (>1% of total OTUs) and the indexes of Chao and Shannon are presented in appendices figures 9 and 10, respectively. No significant differences were observed across the intervention sessions (no-, low- and high- spices).

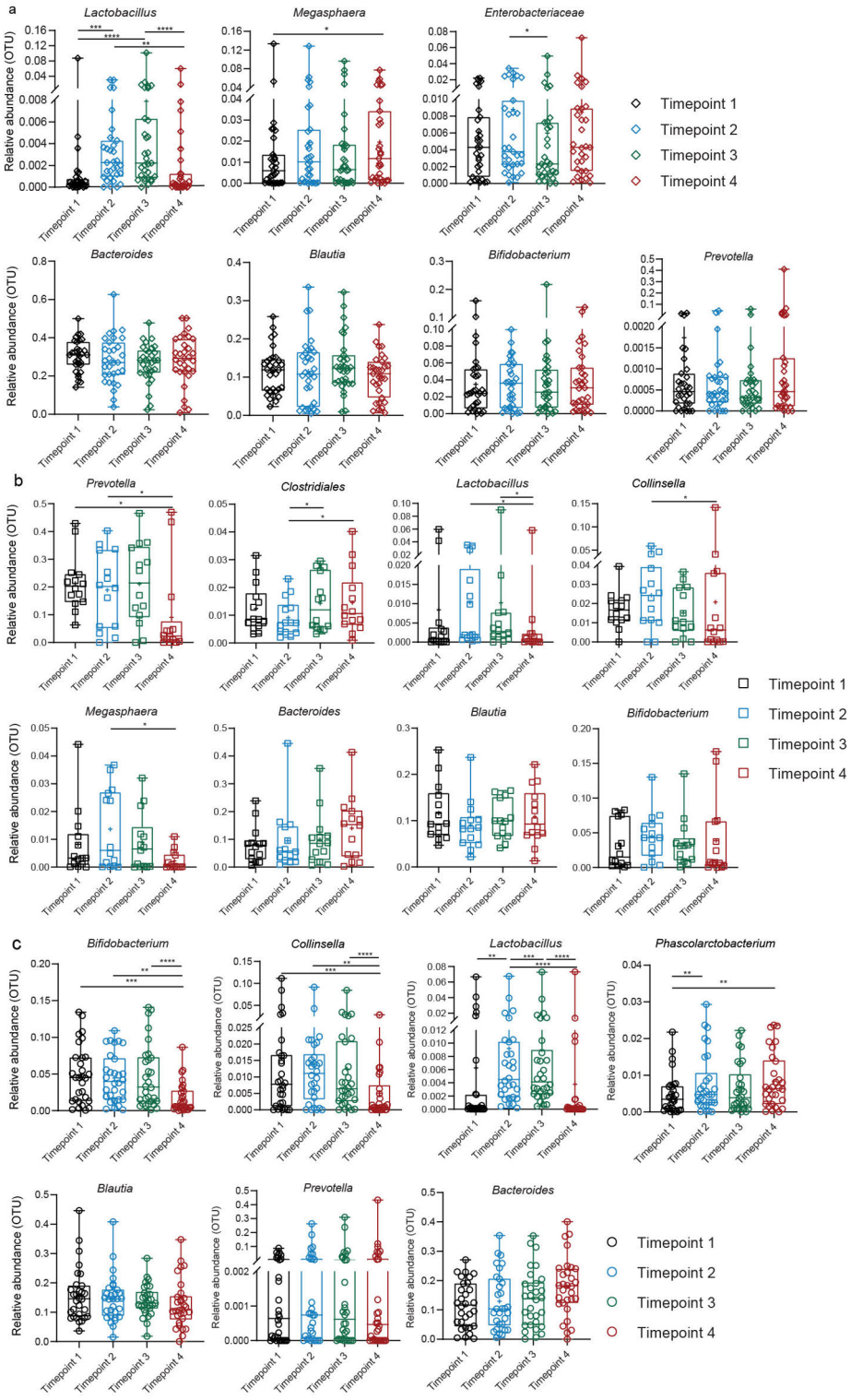
5.2.2 Faecal microbiota changes due to the dietary intervention (Study III, and IV)

5.2.2.1 Faecal microbiota clusters and microbial diversities after consumption of *Lactobacillus* containing fermented milk (Study III) (highlight for aim no. 3. b)

The relative abundances of *Lactobacillus* were less than 1% of the total OTUs in all clusters before consumption. After a once-a-day consumption for seven days (Timepoint 2), the percentage was raised significantly by 1.6 times in Cluster 1 and Cluster 3 (Fig. 11a, c). Although *Lactobacillus* in Cluster 2 is higher than at Timepoint 1, it was not statistically significant (Fig. 11b). It was also maintained at the same level at Timepoint 3 in Cluster 2 and 3. *Lactobacillus* in Cluster 1 increased further at Timepoint 3. However, it decreased twice in Cluster 1 and 2 and 1.5 times in Cluster 3 after cessation of consumption.

Bacteroides were the genera with the highest abundance throughout all the time points in Cluster 1 but, there were no statistical differences between the time points. *Prevotella* and *Blautia* were the top genera before and during consumption of the fermented milk in Cluster 2 and Cluster 3 but were replaced by *Bacteroides* at Timepoint 4 (14 days after cessation of consumption). Apart from *Lactobacillus*, only the abundance of *Megasphaera* in Cluster 1 was significantly changed in an increasing trend compared to Timepoint 1 and Timepoint 4 (Fig. 11a). In Cluster 2, only *Prevotella* was reduced statistically ($p= 0.01$) before and after consumption (Timepoint 1 vs. Timepoint 4) (Fig. 11b). Variation of three genera was found in Cluster 3, where *Bifidobacterium* and *Collinsella* had a reducing trend and *Phascolarctobacterium* an upward trend from Timepoint 1 to Timepoint 4 (Fig. 11c).

Figure 11. ► Relative abundances of the major faecal bacterial genera in the three clusters: (a) Cluster 1 (b) Cluster 2, and (c) Cluster 3 across the time points. Timepoint 1= baseline; 14 days after washout, Timepoint 2= first seven days after ingestion, Timepoint 3= second 7 days after ingestion, Timepoint 4= follow-up; 14 days after cessation of ingestion. (Study III)


a
Lactobacillus
Megasphaera
Enterobacteriaceae
Bacteroides
Blautia
Bifidobacterium
Prevotella
Relative abundance (OTU)
Timepoint 1
Timepoint 2
Timepoint 3
Timepoint 4
b
Prevotella
Clostridiales
Lactobacillus
Collinsella
Megasphaera
Bacteroides
Blautia
Bifidobacterium
c
Bifidobacterium
Collinsella
Lactobacillus
Phascolarctobacterium
Blautia
Prevotella
Bacteroides

Study III investigated the effects of ethnicity, gender, and stool consistency on the faecal microbiota. The faecal microbiota of Chinese people (n=61 out of 75): the major ethnic group, in three different clusters, were significantly separated from each other (Appendix Fig. 9). The minor ethnic groups were not considered in the evaluation of ethnicity because of the small sample size. The faecal microbiota abundances of female and male subjects were randomly distributed among the clusters at all times (Appendix Fig. 10). Generally, the participants experienced healthy stools of types 3 and 4. The faecal microbiota abundances of Type 4 were significantly different in Cluster 1 from Cluster 2 and Cluster 3 at baseline and at Timepoint 3 and were also different from Cluster 1 and Cluster 3 at Timepoint 4 (Appendix Fig. 11).

The alpha diversity indices of faecal microbiota at all time points and clusters were equivalent statistically, except the Chao 1 abundances at Timepoint 3 of Cluster 3 were higher than at Timepoint 2 (Appendix Fig. 12). The beta diversity using unweighted and weighted UniFrac distances for all the clusters were significantly different at all the time points except between Timepoint 3 and 4, and Timepoint 1 and 2 in both distances of Cluster 1 and Cluster 3 (Appendix Fig. 13).

5.2.2.2 Faecal microbiota composition, their changes, and microbial diversities in the dietary intervention using mixed spices (Study IV) (highlight for aim no. 4)

The mixed spices intervention resulted in no significant differences in the relative abundances of the major (>1% of total OTUs) bacterial genera on day two after three doses of spices (Appendix figure 7 d-f). However, the relative abundance changes of two major genera from Day 0 to Day 2 showed differences between the three spices intervention sessions (Fig. 12). The relative abundance changes of *Bacteroides* from Day 0 to Day 2 were significantly reduced at higher spices doses (D1C and D2C) compared with the control (D0C). This can be seen in appendix figure 14, showing that the relative abundances on Day 2 of both D1C and D2C spices were reduced compared to Day 0. Moreover, there was a significant correlation between the relative abundance of *Bacteroides* on Day 0 and their change from Day 0 to Day 2 of the D2C spice session (Appendix Fig. 15). D2C was more sensitive to the high Day 0 count of *Bacteroides*. Regarding *Bifidobacterium*, their relative abundances were increased on Day 2 of the two spices sessions (D1C and D2C) (Appendix Fig. 14), therefore the changes from Day 0 to Day 2 showed a significant increase, especially when compared to D0C and D2C (Fig. 12). Its relation between the relative abundance of Day 0 and the day changes affected the low *Bifidobacterium* to D0C and D2C (Appendix Fig. 15). The alpha diversity using the indexes of Chao and Shannon for the three doses of spices were similar on Day 2 (Appendix Fig. 8).

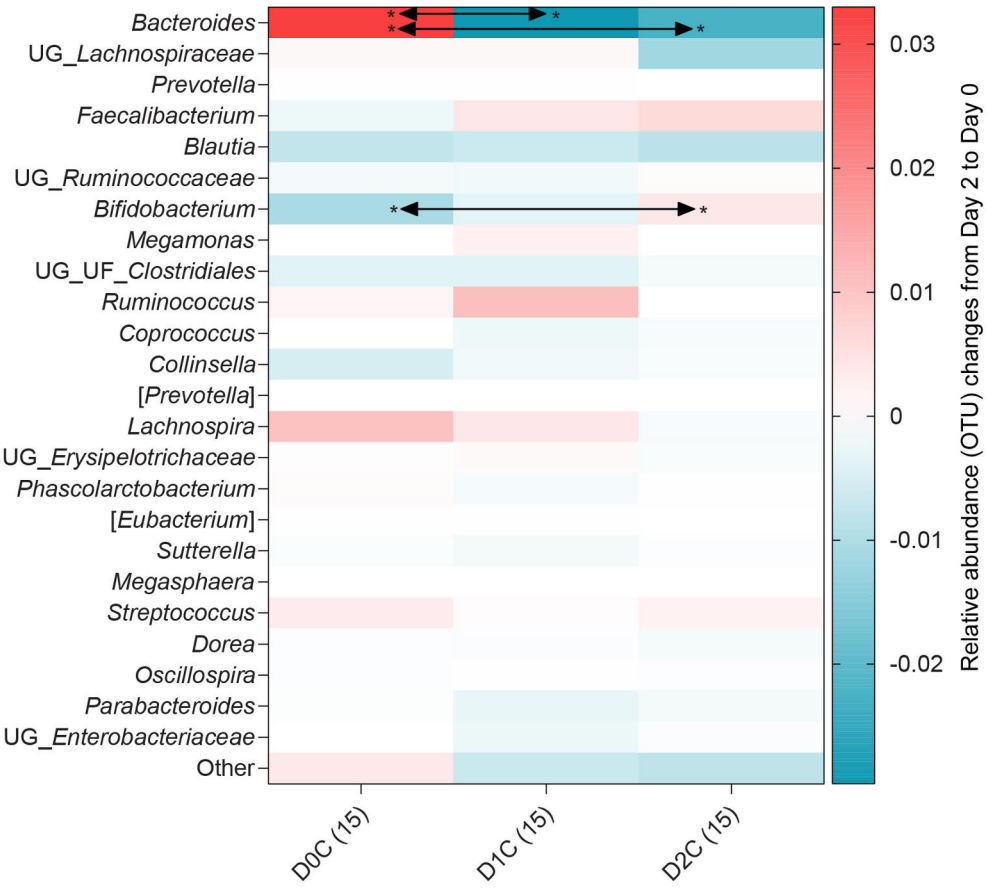


Figure 12. Relative abundance changes of the major faecal bacterial genera after two days of a mixed spices intervention. The heatmap shows the medians of the relative abundance changes from Day 0 to Day 2 for an intervention of three doses of spices. *Adjusted p≤ 0.05. D0C= Meal with no spice intervention, D1C= Meal with low (6 g) mixed spices intervention, D2C= Meal with high (12g) mixed spices intervention, UG= Unknown genus, UF= Unknown family. (Study IV)

5.2.2.3 Correlation of changes in faecal bacterial genera and plasma phenolic acids and changes in total polyphenol excretion (Study IV)

The correlation of three measurements (urinary total polyphenol excretion, plasma cinnamic acid, and plasma phenyl acetic acid) and the relative abundance of bacterial genera changes are summarised in Table 3. Plasma cinnamic acid on Day 1 was strongly associated with the relative abundance changes of *Bifidobacterium* from Day 2 to Day 0. In contrast, *Ruminococcus*, *Bacteroides,* and *Oscillospira* changes were negatively associated with all three measurements. The plasma phenyl acetic acid concentration on Day 1 was negatively correlated with *Phascolarctobacterium*,

Bacteroides, and *Lachnospira*. However, a significant negative association was found between the changes in total urinary polyphenol excretion from Day 1 to Day 0 and relative abundance changes of *Coprococcus* (p= 0.03).

Table 3. The correlation between changes in the 24 hours total urinary polyphenol excretion, plasma cinnamic acid $AUC_{0\text{-}7h,}$ and plasma phenyl acetic acid $AUC_{0\text{-}7h}$ and relative abundance changes of the bacterial genus from Day 0 to Day 2 in the mixed spices intervention. (Study IV)

	Changes in total urinary polyphenol excretion (Day 1 minus Day 0)		**Plasma cinnamic acid $AUC_{0\text{-}7h}$ Day 1**		**Plasma phenyl acetic acid $AUC_{0\text{-}7h}$ Day 1**	
Bacterial genera	**r**	**p values (two-tailed)**	**r**	**p values (two-tailed)**	**r**	**p values (two-tailed)**
Bacteroides	-0.173	0.224	-0.300	*0.038	-0.299	*0.033
UG_*Lachnospiraceae*	0.104	0.467	-0.097	0.512	-0.052	0.717
Prevotella	0.186	0.192	0.075	0.613	0.015	0.914
Faecalibacterium	0.098	0.493	-0.189	0.199	0.133	0.354
Blautia	-0.260	0.065	0.013	0.929	-0.055	0.703
UG_*Ruminococcaceae*	-0.050	0.725	-0.019	0.900	0.187	0.189
Bifidobacterium	0.076	0.598	0.372	**0.009	0.222	0.117
Megamonas	0.137	0.339	0.183	0.214	-0.053	0.714
UG_UF_*Clostridiales*	-0.220	0.121	0.179	0.224	-0.063	0.661
Ruminococcus	-0.007	0.961	-0.308	*0.033	-0.016	0.909
Coprococcus	-0.298	*0.034	0.122	0.411	0.153	0.283
Collinsella	-0.033	0.818	0.269	0.065	0.255	0.071
[Prevotella]	0.065	0.649	-0.254	0.082	0.181	0.205
Lachnospira	0.167	0.243	-0.255	0.081	-0.284	*0.043
UG_*Erysipelotrichaceae*	-0.128	0.370	-0.087	0.556	-0.194	0.173
Phascolarctobacterium	-0.205	0.149	-0.136	0.355	-0.354	*0.011
[Eubacterium]	-0.231	0.102	-0.146	0.322	0.062	0.667
Sutterella	-0.032	0.825	0.272	0.061	-0.022	0.879
Megasphaera	0.133	0.353	0.226	0.122	0.192	0.177
Streptococcus	-0.140	0.329	0.112	0.449	-0.194	0.173
Dorea	-0.255	0.071	-0.239	0.101	-0.068	0.635
Oscillospira	-0.062	0.664	-0.288	*0.047	0.112	0.435
Parabacteroides	-0.068	0.637	-0.153	0.298	0.044	0.761
UG_*Enterobacteriaceae*	-0.004	0.978	0.029	0.843	0.096	0.504
Other (<1% of total bacteria)	-0.165	0.248	-0.141	0.340	0.089	0.533

All intervention doses were combined. Non-parametric Spearman correlation coefficient (r) and two-tailed p values were shown. ** p ≥ 0.001 - < 0.01, * p ≤ 0.05. AUC= Area under curve, UG= Unknown genus, UF= Unknown family.

5.3 Immunological markers: cytokine profiles and bile acids composition in faecal water

Eleven cytokines: IL-1β, -2, -4, -5, -6, -8, -10, -12, GM-CSF, IFN-ɣ, and TNF-α were measured in the faecal water of children (Study I) and adults (Study III). Study I observed the level of cytokines throughout the age groups of the children from the neonatal period (less than one-month-old) to 48 months in order to compare the immunological status of children, especially before and after weaning. Study III examined the level of cytokines in different clusters of the faecal microbiota of adults to investigate the effect of faecal microbiota on the immunological markers.

5.3.1 Comparison of faecal water cytokines in children (Study I) (highlight for aim no. 1. b)

Generally, the faecal water cytokine levels were low, especially before weaning (less than six months) and 24-48 months (Appendix Fig. 16). The level of regulatory cytokines: IL-1β, -2, -5, -8, -12, TNF-α and IFN-ɣ started to increase after the age of six months with an exceptional peak of IL-1β in the 1-3 months group. Afterward, the seven cytokines reached their peak at 12-24 months and faded after 24-48 months. The levels of the anti-inflammatory cytokines: IL-4, -6, -10, and GM-CSF were low across all ages. Levels of IL-1β, -5, -8, -12, TNF-α, and IFN-ɣ varied among all age groups, mainly during weaning periods (comparing before six months groups and 12-48 months groups) (Appendix table 3). IL-12 exhibited the most significant differences between the age groups.

5.3.2 Comparison of faecal water cytokines in faecal microbiota clusters of adults after the introduction of *Lactobacillus* (Study III) (highlight for aim no. 3. a)

Overall, in all three clusters (Cluster 1, 2, and 3), IL-1β, -2, -8, -12, and TNF-α were detectable, while the levels of IL-4, -5, -6, -10, IFN-ɣ, and GM-CSF were near to the detection level or in low amounts at all time points (Appendix Fig. 17). At baseline, the former five detectable cytokines found in Cluster 1 were detected at the lowest concentration among the three clusters. Their levels in Cluster 2 and 3 were 3-6 times and 0.5-6 times higher than those of Cluster 1. IL-2, -12, and TNF-α were the highest in Cluster 2 at baseline, while IL-1β and -8 were detected most in Cluster 3. Before the *Lactobacillus* consumption, higher lymphocytic and pro-inflammatory activities occurred in Cluster 2 and 3. No gender differences were found between the clusters at any time points.

After *Lactobacillus* consumption at Timepoint 2, most cytokines, apart from IL-8, -12, TNF-α, and GM-CSF, were increased. IL-1β had a surge at Timepoint 2, and

IL-2 increased steadily after consumption until Timepoint 4. All faecal water cytokines, including the anti-inflammatory cytokines: IL-4, -6, -10, and GM-CSF, were increased after cessation of the *Lactobacillus* administration (Timepoint 4) compared to Timepoint 1 in Cluster 1(Appendix table 4).

In Cluster 2, IL-1β, -8, and TNF-α levels increased, and IL-2 and -12 were decreased at Timepoint 2. All the cytokines mentioned showed a similar trend at Timepoint 4 except IL-8, which was reduced by 3.8-fold, and IL-1β with the highest surge of 7.2 fold at Timepoint 4. After cessation of *Lactobacillus* consumption, most cytokines such as IL-1β, -4, -10, GM-CSF, and TNF-α were detected as being higher than at baseline.

Interestingly, all cytokines, except IL-6 and GM-CSF, were reduced after consumption at Timepoint 2. Once consumption was stopped, IL-2, -4, -5, -12, TNF-α, and GM-CSF were increased. The level of IL-8 gradually decreased from the baseline to Timepoint 4. However, the level of IL-1β was double at Timepoint 3 compared to Timepoint 1, but was reduced at Timepoint 4.

5.3.3 Comparison of faecal water bile acids in children (Study I) (highlight for aim no. 1. b)

The concentrations of 17 bile acids were measured in faecal water samples of children and compared before and after the weaning period (6-12 months group) (Appendix Fig. 18). Primary and secondary bile acids were prevalent separately before and after the weaning period. Primary bile acids were measurable in the first three months of life and reached their peaks during the 3-13 months period and gradually decreased afterward. In contrast, a minimum of secondary bile acids was detected during the 6-12 months period and then increased and saturated until 48 months. A surge in the remaining bile acids can be seen at 1-3 months, but their concentrations were low in the rest of the age groups.

5.4 Dietary consumption habits and their effect on faecal microbiota

5.4.1 Dietary consumption and intakes in children (Study I and II) and adults (Study III and IV)

The children in Study I were generally introduced to solid foods around 6-12 months of age. They consumed various food items, and ten types of food categories were recorded in daily consumption. Meats, vegetables, dairy products, and beverages were consumed more frequently by the 24-48-month-old children than the younger children (6-12 months) (Appendix Fig. 19).

Cluster 1 children (7-12 years old) consumed more vegetables, dairy products, fruits, plant-based proteins, eggs, seafood, caffeinated drinks, biscuits, Yoghurt, fermented foods, and curry, whereas Cluster 2 children consumed more complex carbohydrates, oily foods, sugar, and meats (Data not shown).

The food frequency questionnaires were recorded 14 days before the washout period in the study on *Lactobacillus* which included the fermented milk intervention. The adults in Study III consumed significantly different food types (Appendix table 5). Cluster 3 consumed four out of the six food types: carbohydrate-rich foods, protein-rich foods, fruits, and nuts, being the most frequent among the three clusters. The weekly consumption of carbohydrates, proteins, and beverages was significantly higher in Cluster 3 than Cluster 2 (Appendix table 5). This suggested that Cluster 3 ate a large variety of foods and a mixture of Western and Asian foods. Cluster 2 had the lowest frequency of food consumption (one out of six food types), but they consumed vegetables significantly more frequently than the other clusters (Appendix table 5). This highlights that the diet of Cluster 2 was a typical plant-based Southeast Asian type (vegetables). Beverages were the most commonly consumed foods in Cluster 1, and carbohydrates consumption was significantly higher than Cluster 2. The frequency of food consumption in Cluster 1 followed second among the three clusters except for beverage consumption, so there is no significant difference in the food types consumed between Cluster 1 and 3.

The dietary intake of energy and nutrients of the adults in Study IV is presented in the appendices table 6. The food dairy intake was recorded for three days before the consumption of the spices and assurances were given that the participants had not consumed polyphenol-containing foods in the run-in period. The energy and nutrients consumed were comparable between the interventions.

5.4.2 Correlation of diets and faecal microbiota of children (Study I and II) and adults (Study III) (highlight for aim no. 1. b, 2 and 3)

The abundances of the major faecal microbiota of the weaned children in Study I (6-48 months old) were highly associated with their diets and found many different associations at different ages (Table 4). Generally, more positive associations between bacterial genera and the food types consumed were found in the younger age groups (6-12 months). Carbohydrate intakes correlated positively with *Clostridiales*, *Ruminococcaceae*, *Phascolarctobacterium*, *Lachnospiraceae*, *Lachnospira* (6-12 months group), and *Ruminococcus*, *Akkermansia*, *Succinivibrio*, *Bacteroides* (12-24 months group). Negative correlations between *Bifidobacterium* and *Phascolarctobacterium*, and complex carbohydrate intakes were observed at 6-12 months and 12-24 months, respectively. Complex carbohydrates seemed to affect

the abundance of bacterial genera in the younger age groups (6-12 and 12-24 months only). Dairy products positively influenced *Akkermansia*, *Bifidobacterium*, *Collinsella*, *Parabacteroides*, *Megasphaera*, and *Lachnospiraceae* in the 24-48 months group. The frequency of fruit consumption induced an opposite trend with abundances of *Phascolarctobacterium* (6-12 months), *Lactobacillus*, *Bifidobacterium* (12-24 months) and *Parabacteroides*, *Ruminococcaceae*, *Akkermansia* (24-48 months) and a positive trend with the abundances of *Ruminococcus*, *Coprococcus* (12-24 months). *Bifidobacterium* and *Bacteroides* were significantly associated with a few food types in the different age groups, whereas *Prevotella* was not correlated with any bacterial genera in all three age groups. *Bifidobacterium* had a negative association with complex carbohydrates (6-12 months), fruits (12-24 months), and dairy products (24-48 months). *Bacteroides* showed a positive association with meats (6-12 months) and carbohydrates (12-24 months) but a negative association with fast foods (mainly fried chicken) at 6-12 months.

Table 4. Correlation between the abundance of faecal bacterial genus (> 1% of total OTUs) and the amount of certain food groups children consumed per day at ages (a) 6-12 months, (b) 12-24 months, and (c) 24-48 months. (Study I).

(a) 6-12 months old age										
Bacteria	Complex Carbohydrates (g)	Vegetables (g)	Legumes and Soys (g)	Fruits (g)	Meats (g)	Dairy Products (g)	Snacks (g)	Beverages (g)	Fast Foods (g)	Seasoning (g)
Bifidobacterium	*-0.661	-0.241	-0.465	0.159	-0.196	0.023	0.159	0.597	**0.765	**0.807
Bacteroides	0.333	-0.059	0.172	-0.041	*0.733	-0.077	-0.296	-0.159	**-0.770	-0.462
Prevotella	-0.137	0.173	0.451	0.255	-0.419	-0.027	0.118	-0.018	0.234	0.098
Faecalibacterium	0.292	0.392	0.372	0.200	0.219	-0.301	-0.346	-0.364	-0.313	-0.364
UG_Lachnospiraceae	*0.692	-0.128	0.065	-0.492	0.009	-0.456	0.082	0.027	-0.214	-0.117
Blautia	0.606	0.542	-0.154	-0.223	0.187	-0.296	*0.697	-0.569	-0.124	-0.205
UG_Ruminococcaceae	*0.706	0.187	0.070	-0.551	-0.378	-0.323	0.023	-0.123	-0.084	-0.121
UG_Enterobacteriaceae	-0.583	-0.091	-0.023	0.583	-0.046	-0.182	-0.528	0.583	0.169	0.494
Klebsiella	-0.036	0.164	0.563	0.237	-0.328	0.428	-0.319	-0.282	-0.452	-0.536
[Ruminococcus]	0.456	-0.118	-0.484	-0.528	0.200	-0.137	0.064	-0.009	-0.258	0.014
UG_UF_Clostridiales	*0.715	0.542	-0.167	-0.314	0.187	*-0.706	0.351	-0.205	-0.065	0.140
Collinsella	-0.241	-0.478	-0.233	0.096	0.059	0.032	-0.123	0.251	0.114	0.051
Coprococcus	0.456	0.155	-0.242	-0.592	0.018	0.191	0.164	-0.446	-0.199	-0.191
Lactobacillus	-0.515	-0.333	0.279	0.333	0.050	0.551	-0.196	-0.077	-0.060	-0.238
Streptococcus	0.059	-0.278	-0.419	-0.506	-0.451	0.132	0.059	0.278	0.303	0.256
Ruminococcus	0.264	-0.146	0.321	-0.237	0.128	-0.191	-0.364	0.082	-0.164	-0.084
Megasphaera	0.146	0.501	*0.628	0.510	0.337	-0.219	-0.182	-0.273	*-0.636	-0.289
Akkermansia	-0.272	0.454	-0.083	0.291	-0.263	0.043	-0.244	0.110	-0.005	0.396
[Eubacterium]	0.100	0.146	-0.098	0.128	0.137	*-0.674	0.100	0.273	0.268	0.443
UG_Erysipelotrichaceae	0.460	0.342	0.070	-0.169	-0.014	-0.132	**0.788	-0.569	0.094	-0.354
Veillonella	-0.273	-0.528	0.233	0.128	-0.191	-0.027	-0.538	0.465	0.219	-0.042
Succinivibrio	-0.347	-0.100	0.445	0.379	-0.571	0.233	-0.210	0.233	-0.027	0.035
Enterococcus	0.137	0.128	-0.079	-0.237	0.091	-0.210	0.091	0.173	0.119	0.308
Dorea	0.454	0.107	-0.124	-0.285	-0.094	-0.340	0.130	0.094	-0.219	0.150
Megamonas	0.132	0.097	-0.454	-0.221	0.201	-0.430	0.325	0.241	0.263	0.598
Phascolarctobacterium	*0.696	-0.104	-0.421	** -0.78	0.293	-0.238	-0.214	0.030	-0.428	-0.036
UG_Clostridiaceae	0.000	-0.273	0.284	0.082	0.091	*-0.073	-0.538	0.146	-0.253	-0.131
Lachnospira	*0.633	0.396	0.181	0.050	0.351	-0.743	0.087	-0.169	-0.378	-0.163
Parabacteroides	0.208	0.153	-0.175	-0.263	0.103	-0.048	0.144	-0.071	-0.139	0.215

(b)12-24 months old age

Bacteria	Complex Carbohydrates (g)	Vegetables (g)	Legumes and Soys (g)	Fruits (g)	Meats (g)	Dairy Products (g)	Snacks (g)	Beverages (g)	Fast Foods (g)	Seasoning (g)
Bifidobacterium	0.322	0.007	0.491	**-0.748	0.497	-0.273	-0.070	-0.140	0.068	-0.296
Bacteroides	*0.629	-0.252	0.516	-0.559	-0.147	-0.280	-0.476	-0.503	-0.196	-0.049
Prevotella	-0.119	-0.042	-0.174	0.000	0.231	0.014	0.322	0.007	-0.231	-0.056
Faecalibacterium	-0.133	-0.259	-0.196	0.524	0.021	0.063	0.413	0.084	0.046	0.134
UG_Lachnospiraceae	-0.168	-0.133	-0.082	0.357	-0.112	-0.070	-0.140	0.056	-0.399	-0.197
Blautia	-0.231	-0.308	-0.125	0.378	-0.133	0.277	0.280	-0.021	-0.288	0.000
UG_Ruminococcaceae	-0.280	-0.343	-0.256	0.469	-0.063	0.116	0.301	0.133	-0.342	-0.070
UG_Enterobacteriaceae	-0.042	-0.007	-0.018	-0.287	-0.266	-0.123	-0.385	0.329	0.000	0.148
Klebsiella	-0.301	0.168	-0.445	0.476	-0.524	0.067	-0.182	0.364	0.139	0.472
[Ruminococcus]	0.503	-0.245	0.473	-0.343	-0.406	-0.336	-0.524	-0.364	-0.484	0.014
UG_UF_Clostridiales	-0.357	-0.077	-0.274	0.329	-0.133	-0.077	-0.126	0.301	-0.484	-0.021
Collinsella	0.049	0.105	0.267	0.133	-0.028	-0.116	0.119	-0.077	0.075	0.359
Coprococcus	-0.287	-0.203	-0.481	*0.636	-0.091	0.126	-0.049	0.315	0.110	-0.070
Lactobacillus	0.413	-0.266	0.445	**-0.783	0.399	-0.399	-0.245	-0.245	-0.274	-0.387
Streptococcus	-0.021	0.196	0.238	-0.280	0.007	-0.578	*-0.671	0.476	0.224	0.021
Ruminococcus	*-0.699	0.084	*-0.655	**0.762	0.154	0.434	0.455	0.406	0.242	0.211
Megasphaera	0.147	-0.070	0.384	-0.469	0.042	-0.438	-0.182	0.196	-0.117	-0.387
Akkermansia	*0.696	0.007	*0.594	-0.580	0.174	-0.192	-0.058	*-0.711	0.048	-0.146
[Eubacterium]	-0.056	-0.105	-0.021	0.000	-0.175	0.312	-0.007	-0.119	0.199	0.570
UG_Erysipelotrichaceae	0.147	0.280	0.352	-0.315	0.392	-0.308	-0.154	-0.154	-0.146	-0.232
Veillonella	0.084	-0.014	-0.068	-0.441	0.112	0.109	-0.245	-0.063	0.285	0.000
Succinivibrio	*0.671	0.031	*0.730	-0.468	-0.491	*-0.594	-0.172	-0.242	-0.119	0.031
Enterococcus	-0.130	0.140	0.016	-0.291	0.501	0.154	0.039	0.105	0.164	-0.296
Dorea	-0.252	0.042	0.039	0.315	-0.091	0.196	0.294	0.091	-0.128	0.239
Megamonas	-0.396	0.411	-0.298	0.224	0.317	0.254	0.515	0.105	0.150	0.169
Phascolarctobacterium	*-0.637	**0.787	-0.464	0.057	0.374	0.488	0.146	0.224	0.391	0.523
UG_Clostridiaceae	-0.119	0.021	-0.231	0.175	-0.545	-0.161	*-0.587	0.385	0.182	0.423
Lachnospira	0.210	0.221	0.248	-0.032	0.116	-0.319	0.004	-0.168	-0.059	-0.226
Parabacteroides	0.242	-0.056	0.278	-0.133	-0.095	0.212	0.322	-0.424	-0.451	-0.081

(c) 24-48 months old age

Bacteria	Complex Carbohydrates (g)	Vegetables (g)	Legumes and Soys (g)	Fruits (g)	Meats (g)	Dairy Products (g)	Snacks (g)	Beverages (g)	Fast Foods (g)	Seasoning (g)
Bifidobacterium	0.204	0.587	-0.025	-0.539	0.299	**-0.874	-0.443	0.395	0.610	0.683
Bacteroides	0.192	-0.048	-0.114	-0.096	0.168	0.072	-0.120	-0.048	0.122	-0.366
Prevotella	0.515	0.228	-0.660	0.299	0.299	0.299	0.228	-0.323	-0.220	-0.024
Faecalibacterium	0.156	-0.084	-0.736	0.084	0.132	0.467	-0.084	-0.012	-0.512	-0.366
UG_Lachnospiraceae	-0.431	-0.335	0.190	0.671	-0.048	*0.838	0.575	-0.431	-0.171	-0.244
Blautia	-0.599	0.168	0.507	0.311	0.000	0.120	0.287	-0.096	0.439	0.561
UG_Ruminococcaceae	0.503	0.287	-0.596	*-0.838	0.000	-0.695	**-0.862	0.575	-0.122	-0.049
UG_Enterobacteriaceae	0.036	0.180	0.444	-0.036	-0.275	-0.204	-0.012	-0.132	0.342	0.122
Klebsiella	-0.659	-0.419	0.330	-0.012	0.467	0.132	0.060	0.252	0.317	-0.122
[Ruminococcus]	0.144	-0.120	0.063	-0.048	-0.647	0.096	-0.120	-0.096	-0.415	-0.342
UG_UF_Clostridiales	-0.659	-0.659	0.317	-0.491	-0.156	-0.180	-0.419	0.659	-0.244	-0.366
Collinsella	0.395	0.563	0.038	-0.443	0.252	**-0.874	-0.299	0.228	0.586	0.610
Coprococcus	0.623	0.527	-0.114	-0.048	-0.359	-0.311	-0.072	-0.240	0.024	0.268
Lactobacillus	-0.024	-0.144	0.266	-0.168	*-0.838	-0.072	-0.216	0.048	-0.415	-0.220
Streptococcus	-0.395	0.084	0.596	0.204	0.299	-0.012	0.275	-0.132	0.732	0.317
Ruminococcus	0.228	-0.204	-0.355	-0.707	0.275	-0.515	-0.611	0.587	-0.122	-0.342
Megasphaera	0.554	0.675	-0.217	-0.434	0.410	*-0.747	-0.361	0.157	0.638	0.491
Akkermansia	-0.012	0.307	0.091	*-0.773	-0.135	**-0.920	-0.700	0.650	0.250	0.450
[Eubacterium]	0.084	0.204	-0.203	0.347	0.084	0.228	0.299	-0.252	-0.073	0.268
UG_Erysipelotrichaceae	0.120	0.144	-0.457	0.263	0.216	0.431	0.120	-0.216	-0.098	-0.073
Veillonella	0.719	0.479	0.076	0.335	-0.168	-0.048	0.359	-0.647	0.244	0.171
Succinivibrio	0.061	-0.503	0.195	0.135	*-0.773	0.282	0.135	-0.184	*-0.75	-0.575
Enterococcus	0.216	0.144	0.228	-0.359	-0.096	-0.575	-0.240	0.168	0.244	0.146
Dorea	0.299	0.228	0.165	0.132	-0.395	0.084	0.060	-0.371	0.098	-0.098
Megamonas	0.024	0.216	-0.596	-0.216	**0.886	-0.048	-0.263	0.287	0.366	0.098
Phascolarctobacterium	0.491	-0.036	-0.051	0.275	0.012	0.180	0.323	-0.443	-0.073	-0.293
UG_Clostridiaceae	0.108	-0.563	-0.216	-0.443	-0.156	-0.036	-0.419	0.347	-0.537	*-0.805
Lachnospira	0.024	-0.240	-0.101	0.120	0.599	0.144	0.216	-0.048	0.146	-0.220
Parabacteroides	0.036	-0.060	-0.355	**-0.946	0.252	*-0.755	**-0.874	**0.874	-0.024	-0.073

Spearman correlation coefficient (r) values are tabulated. The significant positive and negative correlations are shaded in a pink or blue colour respectively. The significance level is indicated as follows: **** $p < 0.0001$, *** $p \geq 0.0001$ - < 0.001, ** $p \geq 0.001$ - < 0.01, * $p \geq 0.01$- < 0.05.

In the older children (7-12 years old) in Study II, the correlation coefficients (r) were lower than those found in Study I although several significant correlations were found (Fig. 13). *Bacteroides*, *Faecalibacterium*, *Bifidobacterium,* and *Collinsella* had moderately high (r= ≥± 0.3) coefficients in six food types among the 15 food types and 1% and above major bacterial genera. *Collinsella* was positively correlated with sugar, fruits, curry, and caffeinated drinks and negatively correlated with vegetables. *Bifidobacterium* had opposite correlations with vegetables and oily foods but was directly associated with sugar. Positive correlations were found between *Bacteroides* vs. caffeinated drinks and *Faecalibacterium* vs. curry. Other food types also influenced the abundance of major faecal microbiota in children.

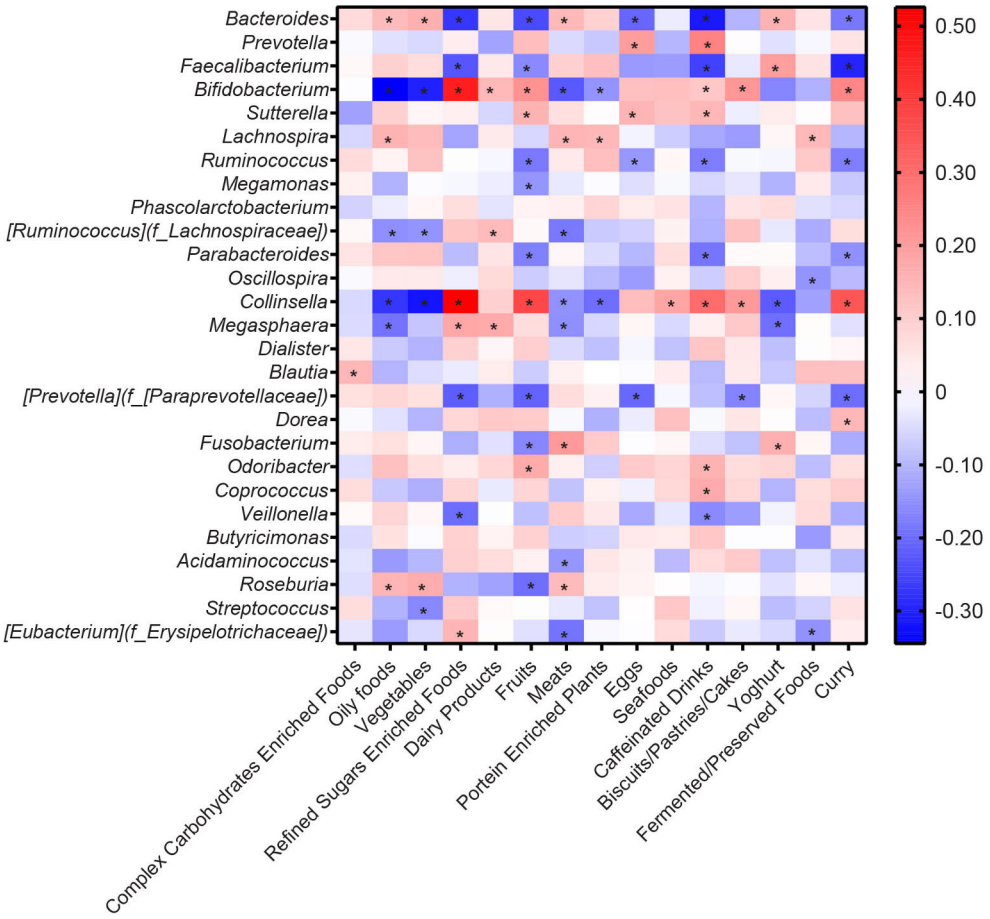


Figure 13. Correlation heatmaps of the quantities of the food types consumed in fractions (X-axis) and relative abundances of faecal bacterial genera (Y-axis). Spearman correlation coefficient (r) values are shaded in a colour gradient. *Two-tailed p ≤ 0.05. (Study II).

As shown in figure 14, the abundance of *Prevotella* was strongly correlated with carbohydrate-rich foods, beverages, nuts, and protein-rich foods, negatively and positively correlated with vegetables. *Blautia* was positively correlated with nuts, protein-rich foods and negatively correlated with vegetables. *Collinsella* was negatively associated with carbohydrates, fruits, and beverages. Consumption of nuts was associated strongly and negatively with a few bacteria, including *Enterobactericeae*, *Lactobacillus,* and positively with *Blautia*. *Bacteroides*, *Ruminococcaceae*, and *Ruminococcus* had no strong correlation with the specific food types.

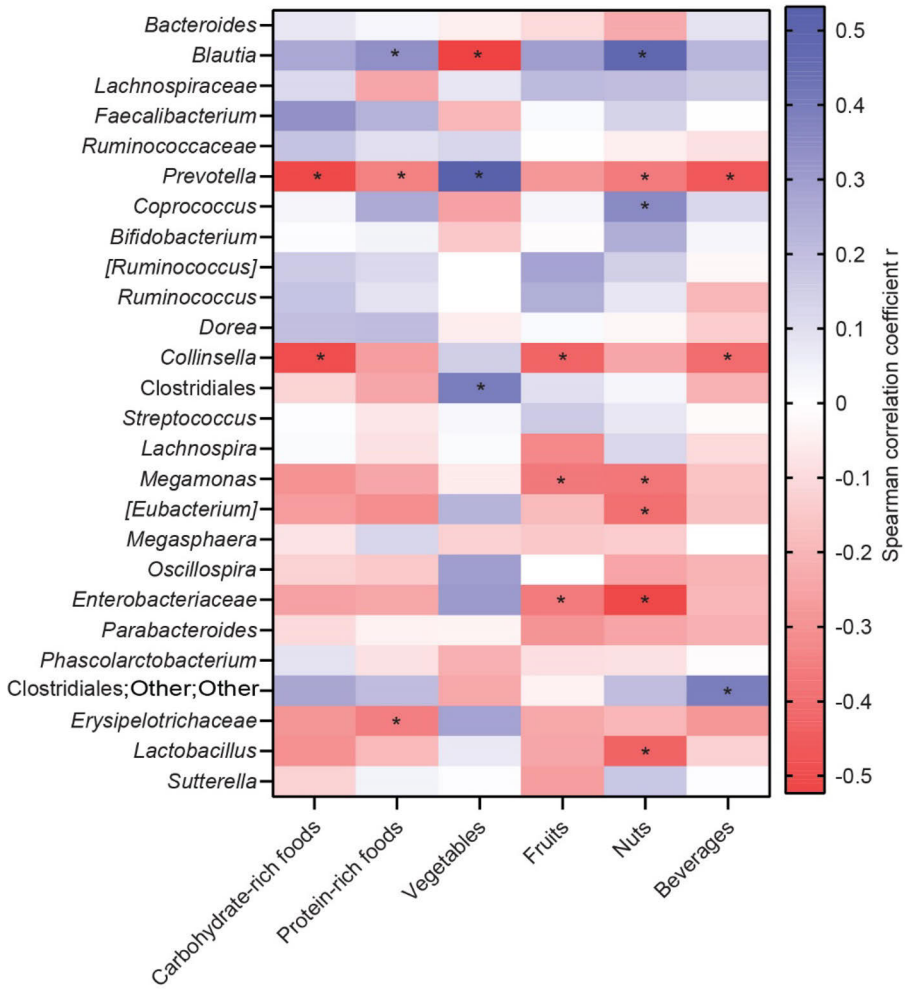


Figure 14. Correlation heatmap of the frequency of food consumption per week (X-axis) and relative abundances of faecal bacterial genera (Y-axis). Spearman correlation coefficient (r) values are shaded in a color gradient. *Two-tailed p ≤ 0.05. (Study III).

6 Discussion

The faecal microbiota of infants differs from that of their mothers (adults) since the composition and functions of the early life microbiome are modulated by external factors such as mode of delivery, gestational age, maternal microbiota, types of feedings, antibiotics consumption, households, living environment, and other issues. During this period, infants grow rapidly, developing their internal organs, immune, metabolic and neurological systems (Polin et al., 2016). The development of the faecal microbiota with age in children is regulated by complex interactions between the host and environmental factors, and *Bifidobacterium*, *Bacteroides*, and *Enterobacteriaceae* have been reported to vary profusely in the faecal microbiota during the early years of a child life (Bäckhed et al., 2015, Laursen et al., 2017).

Study I in this thesis demonstrated that the relative abundances of *Bifidobacterium* and *Bacteroides* were the main two genera among infants' faecal microbiota, and their abundances began to decrease after two years of age. Nevertheless, they remained above one percent of the total OTUs until four years of age. *Enterobacteriaceae* was the third most abundant genera from birth until six months of life, and its abundance then reduced although it remained within 1% of total bacterial count until two years of age. However, the abundances of the adult-type faecal microbiota, *Prevotella*, *Lachnospiraceae*, *Faecalibacterium*, *Blautia*, and *Ruminococcaceae*, gradually reached one percent of the total OTUs in the 6-12 months age group, and *Prevotella* became the top most abundant bacteria at two years of age. Cessation of breast milk feeding and the introduction of solid foods lead to a shift in the faecal microbiota from infantile to adult types (Bäckhed et al., 2015, Stewart et al., 2018). The high dominance of *Prevotella* among Indonesians is probably due to the high consumption of plant-based diets and carbohydrates in Indonesia.

Prevotella was the most prevalent in the faecal and vaginal swabs samples of Indonesian mothers of neonates (Study I). Unexpectedly, in the vaginal microbiota of mothers whose babies were less than a month, *Prevotella* and *Peptostreptococcus* were replaced by *Lactobacillus,* which has been reported to be prevalent during pregnancies (Romero et al., 2014, Aagaard et al., 2012). Their decline after delivery was attributed to the rapid depletion of oestrogens after delivery of the baby and

placenta (Amabebe and Anumba 2018). Despite its high abundance among the mothers' faecal and vaginal microbiota, *Prevotella* remained at a low level in infants until weaning. The meconium and day one stool samples of neonates could reveal whether *Prevotella* or *Lactobacillus* is transmitted during delivery, but we did not examine those samples in Study I. Hormonal changes in mothers after delivery could also explain why the microbiota in the older children's faecal samples shared more similarity with their mothers' vaginal swabs than neonates (less than one month). Nevertheless, after birth, horizontal transmission of microbiota from mothers' vagina to their offspring might happen (Moeller et al., 2018) in older age groups. *Lactobacillus* became the most dominant member of the vaginal microbiota in the non-pregnant Indonesian women (one-month-old and above groups), which is in agreement with other studies and could be dependent on the geographical background (Zhou et al., 2010, Dominguez-Bello et al., 2010, Ravel et al., 2011, Fettweis et al., 2014, Verstraelen et al., 2016).

The breast milk microbiota components of the mothers shared 9-11% features with the faecal microbiota of infants in Study I. *Bifidobacterium* and *Bacteroides* were detected in low amounts in breast milk but prevalent in the infants' faecal samples. The most pervasive bacteria *Staphylococcus* spp. and *Streptococcus* spp. might be commensal because the infants had no known illness. Many studies agreed with this finding (Heikkilä et al., 2003, Hunt et al., 2011, Jost et al., 2013, Moossavi et al., 2019, Lyons et al., 2020).

The transmission of bacteria from mothers, colonisation, and development of faecal microbiota in infants are complex. In Study I, the dominant microbiota of mothers (*Prevotella*) from stool, vagina, and breast milk did not become established as a predominant microbiota in their infants. The bioactive compounds of human milk oligosaccharides (HMOs), which have a bifidogenic effect, enhance the growth of *Bifidobacterium* in the gut (Bouhnik et al., 1999, Katayama et al., 2016, Guarino et al., 2020) before weaning. Among the infantile strains, *Bifidobacterium infantis* could effectively ferment the HMOs from human milk (Asakuma et al., 2011, Rockova et al., 2012). Some *Bacteroides* spp. could involve the digestion of milk by mucus utilisation (Marcobal et al., 2011, Yassour et al., 2018). Exclusive breastfeeding among the Indonesian mothers in this study may favor their continuous growth and suppress the proliferation of *Prevotella*. Regarding the mother-infant bacterial transmission at the strain level, the mother frequently transferred her dominant strain and sometimes secondary strain to her child (Yassour et al., 2018). The Yassour research group proved that the secondary strain of the mother was transmitted to the infant by selecting specific functional genes of the mother, such as a starch utilisation gene (Yassour et al., 2018).

The concentration of the primary bile acids, which aid in the digestion of fat from breast milk (Lyons et al., 2020), was increased in the faeces of infants before

weaning in Study I. Taurine conjugated bile acids are prominent in neonates and adult-type glycine conjugated bile acids are generated gradually when solid foods, especially, vegetables are introduced (Hardison 1978, Karpen & Karpen 2017). Infantile strains of *Bifidobacterium* and *Bacteroides* could survive in the gut, but according to the concentration of bile acids (Kurdi et al., 2006, Watanabe et al., 2017, Tian et al., 2020). These may explain why *Bifidobacterium* and *Bacteroides* were prevalent in infants, which was disclosed in Study I and other studies (Tanaka et al., 2018, Wang et al., 2019, Li et al., 2020). Moreover, Gram-positive Firmicutes including *Bifidobacterium, Clostridium*, *Lactobacillus,* and *Listeria* (Tanaka et al., 2000, Ridlon et al., 2006, Jarocki et al., 2013, Gèrard 2014) and Gram-negative *Bacteroides* (Stellwag & Hylemon 1976, Yoon et al., 2017, Yao et al., 2018) have the activity of bacterial bile salts hydrolases (BSH) which is involved in the deconjugation of conjugated primary bile acids in the small intestine.

We observed in Study I that secondary bile acids were abundant in infants at the age of 12-24 months, which was similar to the studies of Chen, Tanaka, and Faden (Chen et al., 2019, Tanaka et al., 2020, Faden et al., 2021). Meanwhile, dehydroxylation and epimerisation were reported to occur during the 24-36 months period (Tanaka et al., 2020). The conversion of deconjugated primary bile acids into secondary bile acids by microbial 7- alpha-hydroxylase, oxidation, and epimerisation with the help of gut bacteria in the large intestine, co-occur after the introduction of solid foods (Ridlon et al., 2006, Li et al., 2020). Specific species of *Bacteroides*, and *Clostridium* possess the 7-alpha and -beta hydroxylation in the metabolism of bile acids (Ridlon et al., 2006, Gèrard 2014, Yeo et al., 2018). In a study of bile acids from human plasma (Wang et al., 2021), the relative abundance of *Prevotella copri* and *Bacteroides uniformis*, *B. xylanisolvens* were primarily associated with primary bile acids and secondary bile acids, respectively. However, *Bifidobacterium bifidum* and *B. longum* were correspondingly associated with primary bile acids and both (primary and secondary) bile acids (Wang et al., 2021). It can be assumed that some adult-type gut microbiota (*Prevotella*, *Bacteroides*, in Study I) can convert primary bile acids into secondary bile acids but not the infantile *Bifidobacterium*.

The inflammatory cytokine markers from faecal water were determined throughout the age groups of the children in Study I. T helper 1 mediated pro-inflammatory cytokines and chemokine IL-8 were detected around the weaning period (6-12 months) and in two years age group (12-24 months), which may be due to inflammation and facilitated by bacterial or viral infection. This could mark the initiation of immune development. The immune system of infants starts to mature around six months of age and reaches the levels of adults at three years of age (Martin et al., 2010). The observation suggested that the cell-mediated immunity of Indonesian children was active around the weaning period when the diet was

changed. Studies have indicated that *Bifidobacterium bifidum* and *Bifidobacterium*. spp. enhanced the production of the anti-inflammatory cytokine IL-10 (Sun et al., 2020, Hong et al., 2021). In contrast, *Bacteroides fragilis* and *B. ovatus* could prevent lipopolysaccharide-induced inflammation through the manifestation of Th17 and T regulatory cells or anti-inflammatory cytokines production (Tan et al., 2019).

The correlation coefficients between faecal bacterial commensals and potential pathogens were more positive after weaning (6-12 months) than before weaning (Appendix Fig. 1). This implied more interaction between the bacterial species (commensals or pathogens) when solid foods were started, achieving a non-dysbiosis and healthy conditions in infants. In this early stage of life, faecal commensals may inhibit pathogens, or vice versa, before weaning. Interestingly, the presence of *Bifidobacterium* species suppressed the abundance of *Klebsiella* species in the infants' faecal samples before weaning (Appendix fig.1). The same finding was found in vaginally delivered infants in the first year of life (Reyman et al., 2019). This shows the beneficial effects of breast milk and *Bifidobacterium*.

Study II was designed to investigate whether ethnicity or geographic location is the determining factor influencing the faecal microbiota of children (7-12 years old). The faecal microbiota of children from Study II clustered into two distinct groups. *Bacteroides* and *Prevotella* were the most dominant bacteria in Cluster 1 and Cluster 2 regardless of ethnicity or location. Notably, the children from the two clusters consumed different diets.

The children living in the same cities (Kelantan or Penang) in the same cluster could be of different races (Chinese and Malay) and had similar faecal microbiota. In the same city in Malaysia, Chinese and Malay children from the same cluster had similar food types, a notable fusion of foods known as the Peranakan diet (Chinese-Malay). A study comparing Malaysian Chinese, Malay, and indigenous Orang Asli pre-adolescents reported that bacterial diversity and composition of Chinese and Malay were similar but different from those of the tribal children because of the variation in socio-economic conditions (Chong et al., 2015). They concluded that the socio-economic status gap between Chinese and Malays was narrow, and the food types consumed were similar. Our group showed that children's faecal bacterial composition and diversity were similar when comparing two cities in the same country but different from other cities in different countries (Nakayama et al., 2015). The gut microbiota variation depends on the living environment as demonstrated in a mouse study with animals living in the same cage (Lundberg et al., 2017) and a study on genetically unrelated household members (Rothschild et al., 2018). Another study on the Malaysian community of different ethnicities also suggested that the combined effects of the socio-economic factors, including ethnicity, diets, and lifestyles, influence gut microbiota (Dwiyanto et al., 2021).

Some bacteria were distinct among the same ethnicity in the same cluster living in distant cities, such as a comparison of Chinese individuals from two Malaysian cities and Guangzhou or Malays from Kelantan and Penang. *Faecalibacterium*, *Bifidobacterium*, *Collinsella*, *Megasphaera*, [*Prevotella*], and *Fusobacterium* were all different in their abundances compared to the Chinese from Guangzhou and Kelantan in both clusters because some of their foods types were different. Moreover, within Cluster 1, the abundance of *Bifidobacterium*, *Collinsella*, *Fusobacterium*, and [*Eubacterium*] differed between the Chinese from Guangzhou and Penang. Higher bacteria and food diversity were observed between the Chinese from Guangzhou and Kelantan than those from Guangzhou and Penang. This could be explained by Guangzhou and Penang being modern urban cities, but Kelantan being a rural and agricultural area. Although *Odoribacter* and *Butyricimonas* and *Sutterella* and *Streptococcus* differed between the Malays from Kelantan and Penang in both clusters, differences in their foods were not found. However, their socio-economical differences might explain it. This observation strengthens the assumption that the Chinese from both Malaysian cities have similar socio-economic statuses and consume similar food types with similar faecal bacterial profiles.

The bacterial composition and diversity of the children from Bangkok city were found to be lower than those from a rural area of Thailand since the urban children consumed restricted high-fat diets while rural children had broad-base vegetable-based foods (Kisuse et al., 2018). A similar study comparing children from urban and rural areas on an island in the Philippines found that their different enterotypes were classified due to the high-fat dietary habit in the metropolitan city (Nakayama et al., 2017). Previous twins/triplet studies hypothesied that there is a specific genetic influence on the gut microbiota (Murphy et al., 2015, Yang et al., 2020), however, this is not supported by our studies. In the twin studies, monozygotic co-twins shared more gut microbiota than dizygotic twins at birth (Yang et al., 2020). Nevertheless, microbiota differences diminished from month two and became uniform at month 12 (Murphy et al., 2015). This further supports the hypothesis that diet is the determining factor as regards the gut microbiome. Moreover, the twin studies could not exclude that the sharing of the placenta and amniotic fluid in monozygotic twins leads to a similar source for their microbiota.

Nevertheless, it was reported that Han Chinese from different regions within China, Australia, and Hongkong had similar gut bacterial compositions (Prideaux et al., 2013, Lin et al., 2020). Another study of young Han Chinese adults from urban and rural areas of Northern China showed nine predominant genera segregated from non-Han Chinese at the species level according to geographical location (Zhang et al., 2015). *Bacteroides* (Guangzhou Cluster 1 in Study II) were prevalent in third place among 1% of total OTUs, and *Prevotella* (Guangzhou Cluster 2 in Study II) was not a core gut microbiota (Zhang et al., 2015), so the gut microbiota of

Guangzhou (Southern China) children was different from that of Northern Chinese. Interestingly, the *Bifidobacterium* abundances in Guangzhou children among Cluster 1 and Cluster 2 in our study were 0.4% and 0.06% of total OTUs and were the lowest among all the children. *Bifidobacterium* dominated among Beijing and Lanzhou children from Northern China and belonged to the *Bacteroides-Bifidobacterium* enterotype (Nakayama et al., 2015). These findings firmly concluded that ethnicity is not a major determinant of the faecal microbiota profile, but geographic location and socioeconomic conditions are. Different locations impact different diets, dietary habits, socioeconomic statuses, environmental exposures, and lifestyles (cultures). The diets/food types consumed influence the gut microbiota locally.

Studies III and IV were designed for healthy adults with a normal BMI range. Study IV included only Chinese males, whereas Study III was comprised of males and females, mostly Chinese (81% of participants). Before the interventions, both studies excluded the subjects who consumed fermented foods and Indian spices to remove or reduce related confounding factors. The predominant faecal bacterial genera of adults from Singapore (Study III and IV) were *Bacteroides, Lachnospiraceae*, *Blautia*, *Prevotella*, *Faecalibacterium*, *Ruminococcaceae,* and *Bifidobacterium*. *Bacteroides* was the highest abundance. In Study III, three clusters were found. Cluster 1 (*Bacteroides*: 30% of total OTUs); Cluster 2 (*Prevotella*: 21% of total OTUs); and Cluster 3 (enriched *Blautia*, *Faecalibacterium*, *Bacteroides*, *Lachnospiraceae* in order). *Ruminococcaceae* and *Ruminococcus* were more abundant in Cluster 3 than in the other two clusters. The subjects with *Prevotella* from Cluster 2 were in a normal BMI range, although the amount of *Prevotella* has been reported high in Chinese adults (Duan et al., 2021) and middle-aged Spanish subjects (Moreno-Indias et al., 2016) with obesity.

Prevotella has been associated with foods enriched in fibre from plants (Durbán et al., 2013, Ferrocino et al., 2015, Gutiérrez-Díaz et al., 2016, Dubois et al., 2017, Nakayama et al., 2017, De Filippo et al., 2017), which agrees with the result from Study III Cluster 2, *Prevotella* was positively associated with those vegetables which were consumed the most among three clusters. It has been suggested that 19% of Singapore adults from Study III who belong to Cluster 2 (*Prevotella*) had adopted the Southeast Asian dietary habit (Nakayama et al., 2015). *Prevotella* was also linked with the eggs and caffeinated drink consumption of the children in Study II, both of which were highly prevalent in Cluster 2. The children in Study II consumed more oily foods, sugars, fruits, seafood, caffeinated drinks, yoghurt, fat spreads, and curry. In this study, the association effect may have been related to the combination of foods, including high-fibre fruits.

After cessation of breast milk, the children aged between 12-48 months in Study I, had more *Prevotella* and frequently consumed vegetables, dairy products, meats, and beverages; however, an association between *Prevotella* and vegetables could not

be found. The faecal microbiome of children started to develop after weaning but as yet was not stable; meanwhile, the types of food consumed varied from the younger infants to the children at 48 months. *Prevotella* has been suggested to digest complex carbohydrates such as rice and grains (Dubois et al., 2017, Nakayama et al., 2015). Indica rice is the primary source of carbohydrates in Indonesia and has a high resistant starch content (Calingacion et al., 2014, Custodio et al., 2019). Nakayama et al. proposed that *Prevotella* in Indonesian children (Study I) populated the gut after weaning because of the resistant starch from Indica rice which reduces the secondary bile acids in the gut (Hylla et al., 1998, Jenkins & Kendall 2000), however, some of the species of *Prevotella* are sensitive to bile acids (Hayashi et al., 2007, Nakayama et al., 2015).

Association between *Bifidobacterium* and diets has been revealed in many dietary intervention studies. *Bifidobacterium*, a beneficial gut microbe, is mainly found in breastfed infants before their weaning period. Moreover, the plant-based proteins (peas), high-fibre fruits (apple pectin), grains (whole-grain barley, cereals), prebiotics (inulin, galactooligosaccharides), antioxidants; polyphenols (flavanoids: red wine, cocoa, anthocyanins), carotenoids (Lutein), fermented foods (soybeans based) prevalent in the Mediterranean diet have been reported to enrich the growth of *Bifidobacterium* in the human gut (Costabile et al., 2008, Ramnani et al., 2010, Shinohara et al., 2010, Dominika et al., 2011, Tzounis et al., 2011, Dewulf et al., 2012, Hidalgo et al., 2012, Inoguchi et al., 2012, Queipo-Ortuño et al., 2012, Vulevic et al., 2012, Martínez et al., 2013, Molan et al., 2014, Mitsou et al., 2017, Vandeputte et al., 2017). Although Studies III and IV did not find any significant positive correlations between *Bifidobacterium* and foods, the bacteria were associated with seasonings and fast foods (Indonesian fried chicken) in 6-12-month-old Indonesian infants in Study I and fruits, dairy products, caffeinated drinks, pastries, curry, sugars in the children in Study II. Some food compounds from the literature above, especially high-fibre fruits, prebiotics, and polyphenols, might be involved in the foods consumed by children from Guangzhou and the two Malaysian cities (Study II).

Importantly, *Bifidobacterium* from 6-12-month-old Indonesian infants was negatively associated with complex carbohydrates, as the infantile *Bifidobacterium* could not digest resistant starch and its association with bile acids as discussed earlier. Moreover, local fruits and dairy products from Indonesia may have some secondary metabolites with anti-microbial properties, which would remove the infantile *Bifidobacterium* from the guts of infants and their mothers; however, this would need to be further investigated.

Many studies reported that the consumption of animal-based proteins, high-fat and high-sugar diets enrich the growth of *Bacteroides* species in the gut of children and adults (De Filippo et al., 2010, Yatsunenko et al., 2012, David et al., 2014,

Nakayama et al., 2015, De Filippo et al., 2017, Koponen et al., 2021). *Bacteroides* tolerate bile acids in the gut even though high-fat, animal-based diets stimulate bile acid production (David et al., 2014, Koponen et al., 2021). Abundances of *Bacteroides* from Cluster 1 children in Study II were associated with oily foods, meats, yoghurt, and vegetables that might have more digestible carbohydrates, and they consumed more complex carbohydrates, vegetables, meats, plant-based proteins, eggs, biscuits, dairy products, fermented foods than Cluster 2 children. Likewise, meats and carbohydrates were linked with *Bacteroides* after solid foods were introduced to the infants in Study I. It is noteworthy that 41% of Study III adults who belonged to Cluster 1 (*Bacteroides*) and preferred Western and East Asian dietary habits (De Filippo et al., 2010, Yatsunenko et al., 2012, Nakayama et al., 2015), showed a high proportion of beverages, meats, and carbohydrates in Study III. However, *Bacteroides* were not associated with any particular food types.

Cluster 3 adults in Study III (40% of the total subjects) had comparable abundances of major bacterial genera, as they consumed high amounts of both Western and Southeast Asian foods which are the highest in carbohydrates, proteins, fruits, and nuts among the three clusters. It was observed that typical Singaporeans randomly enjoy a variety of foods in rotation (Naidoo et al., 2017). Such an alternating dietary habit could lead to Cluster 3 having no predominant faecal bacteria, unlike Cluster 1 and 2, which both possess a predominant microbiota. *Blautia* was linked directly to nuts but oppositely to vegetables in adults (Study III) and to carbohydrates and snacks in 6-12- months-old infants (Study I) and children (Study II). High-fibre, carbohydrates, and prebiotics, which increase short-chain fatty acids, enhance the concentration of *Blautia* in the mammalian gut (Liu et al., 2021). Healthy eating behaviour was assumed to be related to *Blautia* (Medawar et al., 2021). *Ruminococcus* can degrade mucin (Salyers et al., 1977), carbohydrates, and dietary fibres and is also associated with an animal-based diet (Tomova 2019). Study I confirmed that *Ruminococcus* was correlated with complex carbohydrates and fruits, but Study III did not find any significant associations between *Ruminococcus* and diets. *Collinsella* was found positively related to refined sugars-rich foods and fruits but negatively to vegetables and oily foods in Study II children, and carbohydrate-rich foods in Study III adults. The studies also suggested that *Collinsella* was inversely correlated with fibres and processed foods and related to unhealthy eating behaviour and inflammation (Gomez-Arango et al., 2018, Medawar et al., 2021, Manni et al., 2021).

The interaction between gut bacteria and diets is complex. The results depend on many external and internal factors such as socio-economy, geography, health status, locally available foods, dietary habits, duration of diet consumption, the design of FFQs, and so forth. In the interventions in Study III and IV healthy Singaporean adults were given a probiotics milk drink, and meals containing spices. Bacterial

diversities (alpha and beta) in both studies remained unchanged after the intervention, suggesting that their faecal microbiome was structurally stable throughout the studies.

The *Lactobacillus* in the consumed milk drinks consumed increased its relative abundance in the faecal samples of all three clusters during the 14-day intervention, as shown in Study III. After seven days of the intervention, the relative abundance of *Lactobacillus* was significantly higher than at baseline among Cluster 1 and 3, but the rise stagnated over the next seven days. Cluster 2 did not show a significant increase in *Lactobacillus* during consumption. Once the consumption stopped, the *Lactobacillus* abundance returned to its baseline level in all the clusters in two weeks. Moreover, during the intervention, some faecal microbiota were modulated by *Lactobacillus* consumption in the three clusters. Among the major faecal microbiota, only one (*Megasphaera*) genus and three genera (*Bifidobacterium*, *Collinsella,* and *Phascolarctobacterium*) significantly changed their abundances in Cluster 1 and 3. *Bacteroides* (Cluster 1 enterotype) and *Blautia* (the highest bacteria of Cluster 3) abundances were not altered. However, the abundance of Cluster 2 enterotype, *Prevotella*, was reduced after *Lactobacillus* consumption, but no other major genera were modified. Some strains of *Lactobacillus* prevent the growth of *Prevotella* (Vuotto et al., 2014, Larsen 2017, Mohan et al., 2020).

In Cluster 1 and 3, *Lactobacillus* administration enhanced the growth of endogenous *Lactobacillus* in the gut and modulated another major faecal microbiota without interfering with their enterotypes. Interestingly, in Cluster 2, the impact of exogenous *Lactobacillus* on endogenous *Lactobacillus* was less pronounced, and no modulation of major faecal microbiota was observed apart from *Prevotella* during the *Lactobacillus* intervention. We could not differentiate the *Lactobacillus* strains found in the faeces because the strain-specific primers were not accessible. Nevertheless, it was concluded that *Lactobacillus* could not colonise and persist in the gastrointestinal tract of Singapore young adults for longer than 14 days after cessation of consumption. Crucially, colonisation and recovery of *Lactobacillus* rely on the host's basal microbiota profile and diets. These findings and conclusions are consistent with previous studies (Khine et al., 2019, Hou et al., 2020).

When the immunological responses were compared among clusters before *Lactobacillus* introduction in Study III, pro-inflammatory cytokines could be detected in Cluster 3 and 2. In detail, 8 out of the11 faecal water cytokines, including anti-inflammatory cells, were highest in Cluster 3, while the anti-inflammatory cytokines were least active in Cluster 2. Meanwhile, 6 out of the 11 cytokines, pro-inflammatory cells, were lowest in Cluster 1 at baseline. Cluster 2 had higher pro-inflammatory activities, such as IL-2, -12, and the TNF-α levels were 3-6 times higher than that of Cluster 1 at baseline. Cluster 3 had higher IL-1β, -4, -10, -12, TNF-α cytokines (3-10 times) than Cluster 1 at baseline; these cytokines are involved

in pro- and anti-inflammatory pathways. Due to their basal faecal microbiota, the immunological activities in each cluster behaved differently before *Lactobacillus* administration.

Regarding the immunological changes after seven days of *Lactobacillus* administration, Cluster 2 and 3 showed the highest alteration (up-and down-regulation) of cytokine levels compared to their baselines. In Cluster 2 the pro-inflammatory cytokines: IL-1β, -8, TNF-α, IFN-ɣ; anti-inflammatory cytokines: IL-10; and T-helper 2 cells mediated IL-4, -5 were boosted after seven days of administration. The enhanced levels of anti-inflammatory IL-4 and -10 and inflammatory IL-1β were retained during the follow-up period. Moreover, pro-inflammatory activities in Cluster 2 shifted from neutrophil-mediated IL-8 to leukocytic-mediated IL-1β pathways upon *Lactobacillus* consumption. *Prevotella* was most likely the cause of inflammation, while *Lactobacillus* reduced *Prevotella* and increased the anti-inflammatory cytokines. These processes balanced the cytokines profile. According to a previous report, when specific *Prevotella* species were abundant, IL-1β, -6, -8, -23, TNF-α are stimulated through dendritic and epithelial cells that activate neutrophil production in gut inflammatory diseases, periodontitis, and bacterial vaginosis (Larsen 2017).

However, Cluster 3 down-regulated 9 out of the 11 cytokines such as pro-, anti-inflammatory, and T-helper cells after seven days of *Lactobacillus* consumption but, at the follow-up period, only IL-5 was increased, and the remainder were reduced or marginally increased. Pro-inflammatory responses in Cluster 3 were diminished by the acute down-regulation of cytokines and balanced the cytokines after *Lactobacillus* administration. Cluster 1 showed the minimal alteration, and delayed the up-regulation of all pro-and anti-inflammatory cytokines 14 days after the cessation of *Lactobacillus* (follow-up), except for IL-1β, which was higher at baseline and after seven days of *Lactobacillus* consumption.

The effect of *Lactobacillus* on faecal microbiota was basal microbiome dependent, and the immunological responses caused by changes in faecal microbiota also depended on the basal faecal microbiome. Hence, Study III adults in Cluster 2 and 3 experienced more active immunological modulations, and *Lactobacillus* triggered a more beneficial immunoregulatory effect (anti-inflammation) than Cluster 1. Moreover, the diets discussed above highly influenced the basal faecal microbiota. Since Singapore is a developed, multi-ethnic, and multicultural urban city that in 2019 (the latest available data) had the world's second-longest healthy life expectancy (HALE) at birth (HALE data by country), the choice of diet (broad-based) and dietary habit (random and rotation) could be the benefactors. Many health benefits and diseases have been associated with the major gut microbiome clusters such as *Bacteroides* and *Prevotella*. However, further studies investigating the health status of Cluster 3 adults who had the Singaporean style of alternating dietary habits,

consuming a wide range of dietary composition and an evenly distributed basal faecal microbiome, should be conducted.

Dietary polyphenolic compounds modulate the gut microbiome, and studies have shown the bi-directional association between phenolic compounds and gut bacteria (Duda-Chodak et al., 2015, Espín et al., 2017, Loo et al., 2020). In our dietary intervention study (Study IV), we investigated the effect of polyphenol-rich mixed spices on faecal microbiota in one meal in three separate doses for a short period (24 hours). The cohort in Study IV was similar to Study III in terms of age, race, and residing location, and *Bacteroides*, *lachnospiraceae,* and *Prevotella* were the most prevalent in participating Singapore adults (Study IV).

Spice doses 1 and 2 (D1C and D2C) curries significantly achieved an increase in the relative abundance of *Bifidobacterium* (D2C vs. D0C) and a decrease in the relative abundance of *Bacteroides* (D1C and D2C vs. D0C) from Day 0 to Day 2 of intervention. In contrast, no polyphenol dose control (D0C) showed the opposite trend for both bacteria. The observation further supported the change in the abundance of *Bifidobacterium* on Day 2 was positively correlated with the relative abundance on Day 0 in the high (D2C) and low (D1C) dose of spices interventions but negatively for *Bacteroides* at a high dose only. Nevertheless, these findings could be attributed to a subset of subjects (Appendix Fig. 15), which point toward individual variation and very short-term intervention. This highlights those variations among the cohort depending on the individual basal faecal microbiome and these varied with their respective diet.

Nevertheless, the faecal microbiota was modified within a day of consuming the single dose of curry spices. Other studies have also reported gut bacterial modulation in short period dietary interventions (Wu et al., 2011, David et al., 2014). In our study, the alpha diversities of the faecal microbiome were the same in the three doses of spices before and after consumption. This might also explain why a single administration of spices in a short time frame does not alter the basal niches of bacteria. However, the findings need to be confirmed by studies with a longer duration of interventions, that also investigate microbiome stability and their profile changes.

One in-vitro spice extract study showed that the growth of *Bifidobacterium* spp. and *Lactobacillus* spp. were enhanced (Lu et al., 2017). Lu et al. also reported that 26 OTUs were different after two weeks of an intervention with five mixed spices and the changes included a rise in *Bifidobacterium* spp., *Bacteroides* spp., *Lactobacillus* spp., *Bacteroides*, *Ruminococcus,* and a decrease in *Clostridium* (Lu et al., 2019). These results differed from Study IV, but the participants' basal gut microbiota also differed from Study IV, and other factors such as differences in the intervention period and studied spices, diet, socio-economic condition, and geographic locations could be relevant. Nevertheless, polyphenolic compounds from

different sources of diets, such as red wine (Clemente-Postigo et al., 2013), wild blueberries (Guglielmetti et al., 2013), tart cherries (Mayta-Apaza et al., 2018), and five mixed spices (cinnamon, oregano, ginger, black pepper, and cayenne pepper) (Lu et al., 2019) were shown to enhance the *Bifidobacterium*, and the enhancement was associated with anthocyanins, a secondary metabolite, and phenolic acids (Boto-Ordonez et al., 2014).

All these findings and the results of Study IV indicate that polyphenol spices may have a potential bifidogenic effect and prebiotic properties (Marchesi et al., 2016). In-vitro and in-vivo polyphenols studies have also found that *Bacteroides* could be suppressed by taking chlorogenic, caffeic acid, rutin (Parker et al., 2013), cocoa (Massort-Cladera et al., 2012), tea, and phenolic metabolites (Lee et al., 2006). Furthermore, low *Bacteroides* abundance caused by high doses of spices was also negatively associated with plasma phenolic acids (Cinnamic acid: CNA and Phenyl acetic acid: PAA). However, the high *Bifidobacterium* on Day 2 appeared to be only positively associated with CNA. The intake of the mixed spices was strongly linked with phenolic acids, CNA, and PAA, which were raised by the three different doses of spices and are therefore proposed as plasma biomarkers. (Halder et al., 2018).

As summarised in the Literature Review section, *Bifidobacterium*, *Prevotella*, *Bacteroides*, and *Blautia* are associated with health benefits. The impact of probiotics, *Lactobacillus,* and dietary polyphenols on the gut microbiome and their interactions with diets (especially the Singapore style of alternating dietary consumption) and their health applications lead us closer to developing practical tactics for achieving a healthier personalised gut microbiome.

7 Summary/Conclusions

The Indonesian mothers possessed high abundances of *Prevotella* genera in their faecal and vaginal microbiota and low abundances of *Bifidobacterium* in their breast milk microbiota, and therefore Study I was able to illustrate the nature of the transmission of microbiota from the mothers and their successful establishment in their infants. Maternal basal microbiota, which depends on local foods and dietary habits, was one of the crucial factors influencing the transmission and establishment of microbiota. The faecal microbiota spectrum of infants depended on their age and the dietary change from breast milk to solid foods and adult-type foods. Indonesian infants acquired *Bifidobacterium* and *Bacteroides* in their faeces from one month to two years of age; meanwhile, adult-type bacteria such as *Prevotella* appeared in their faeces after weaning, with the highest abundant at two years of age. The transition of bacterial changes occurred during weaning (6-12 months old) because of the changes in the infants' diet. Survival of *Bifidobacterium* and *Bacteroides* in infants' faecal microbiota was favoured by the HMOs in human milk, exclusively breastfeeding, and a GI environment such as the relative profile of bile acids. The types and concentrations of bile acids and immunological markers such as cytokines in the infants' faecal samples varied according to their faecal microbiota which was related to their ages and diets. The weaning period is a noteworthy milestone for infants replacing from breastfeeding with an adult-type diet, which changed their faecal microbiota, followed by the profile and level of their bile acids and cytokines. This process dramatically impacts the health of infants. Infantile faecal microbiota seemed to inhibit the potential pathogens before weaning, but after weaning with more mature immunity, no apparent health issues were found in infants although more faecal bacterial-host interactions occurred.

Similar faecal microbiota composition was found in the different ethnic children (Chinese and Malay) living in the same city (Penang or Kelantan), indicating that similar food types, socio-economic status, and living conditions in the same residential location are the main factors that contribute to the microbiota profile. This was also confirmed by, different faecal microbiotas being observed in the same ethnic children (Chinese or Malay) from different cities, Penang, Kelantan, and Guangzhou, showing that geographic location but not ethnicity, is the main

determinant of faecal microbiota.–The types of food consumed by children from Guangzhou differed from the other two Malaysian cities; hence, their bacteria diversities were distinct. However, the bacterial diversities between Guangzhou and Penang were less than those between Guangzhou and Kelantan since the socio-economic condition in Guangzhou is comparable to Penang but not Kelantan. Although Malay children from Penang and Kelantan consume similar types of food, their faecal microbiota were different due to the gap in their socio-economic differences. Overall, the studies suggest that ethnicity is not the major factor in determining the gut microbiota of the population, and that geographical location disguises the main influencing factor of local diets.

Singapore adults (Study III) belong to *Bacteroides*-predominant (Cluster 1), *Prevotella*-predominant (Cluster 2), and non-predominant/evenly distributed (Cluster 3) bacterial clusters. Each cluster consumed different diets, such as Westerns/East Asians (Cluster 1), Southeast Asians (Cluster 2), and Westerns/Southeast Asians (Cluster 3). Inflammatory cytokines were more active in Cluster 2 and 3 than Cluster 1. *Lactobacillus* fermented milk consumption modulated faecal microbiota, and subsequently, the immunological responses (cytokines levels) in each cluster differed according to their basal faecal microbiota. A surge of *Lactobacillus* was found after a short period (seven days) of consumption in Cluster 1 and 3 but not in Cluster 2 and the level was not sustained in all the clusters two weeks after the cessation of *Lactobacillus* consumption. Because of the reduction in *Prevotella* by *Lactobacillus* in Cluster 2 in the intervention study, anti-inflammatory cytokines were up-regulated and balanced the inflammatory cytokines' activities. Cluster 1 and 3, after two weeks of *Lactobacillus* consumption, exhibited modulation in some major bacteria compositions but not their enterotypes. Cluster 3 downregulated most of the cytokines and remained stable during the washout, while Cluster 1 alteration in cytokines levels was minimum and delayed but up-regulated. *Lactobacillus* caused a beneficial immunoregulatory effect in Cluster 2 and 3. The Singapore adults in Cluster 3 possessed evenly distributed basal faecal microbiome, due to consuming mixed diets of Westerns and Southeast Asians; this is typical of Singapore's unique multi-ethnic/-culture, which promotes a wide variety of food choices, and random and alternating dietary eating habits.

Abundances of basal major faecal microbiota were modified within a short period (one day) by a meal containing mixed spices (Study IV). The inter-individual variations depended on their respective basal diets and faecal microbiota. Higher abundance of *Bifidobacterium* in interventions with a high dose of spices and lower abundance of *Bacteroides* in interventions with both high and low dose spices were because of the bifidogenic and suppression of *Bacteroides* by polyphenol compounds in the mixed spices.

To obtain a complete and better overview of the microbiome of infants further study needs to be done by employing a longitudinal study covering the ages of infants from the same cohort and comparing the different modes of delivery and other determining factors. To be more specific about the effect of the ethnicity factor on the gut microbiota profile, it would be useful to have more knowledge of human leucocyte antigens (HLA) variants in different races. Dietary intervention studies with a longer duration need to be done on similar dietary and lifestyle practices in the same cohort. Moreover, further knowledge concerning the functional profile of microbiotas would be necessary in order to carry out analysis using metagenomic, metatranscriptomic, metaproteomic and metabolomic approaches. Dietary habits form the primary consideration for studies on basal gut microbiota profiles of different ages in specific geographical locations. Knowledge in applying dietary interventions (pro-prebiotics/diets) is the essential foundation for future personalised microbiome-related supplements for our health and wellbeing.

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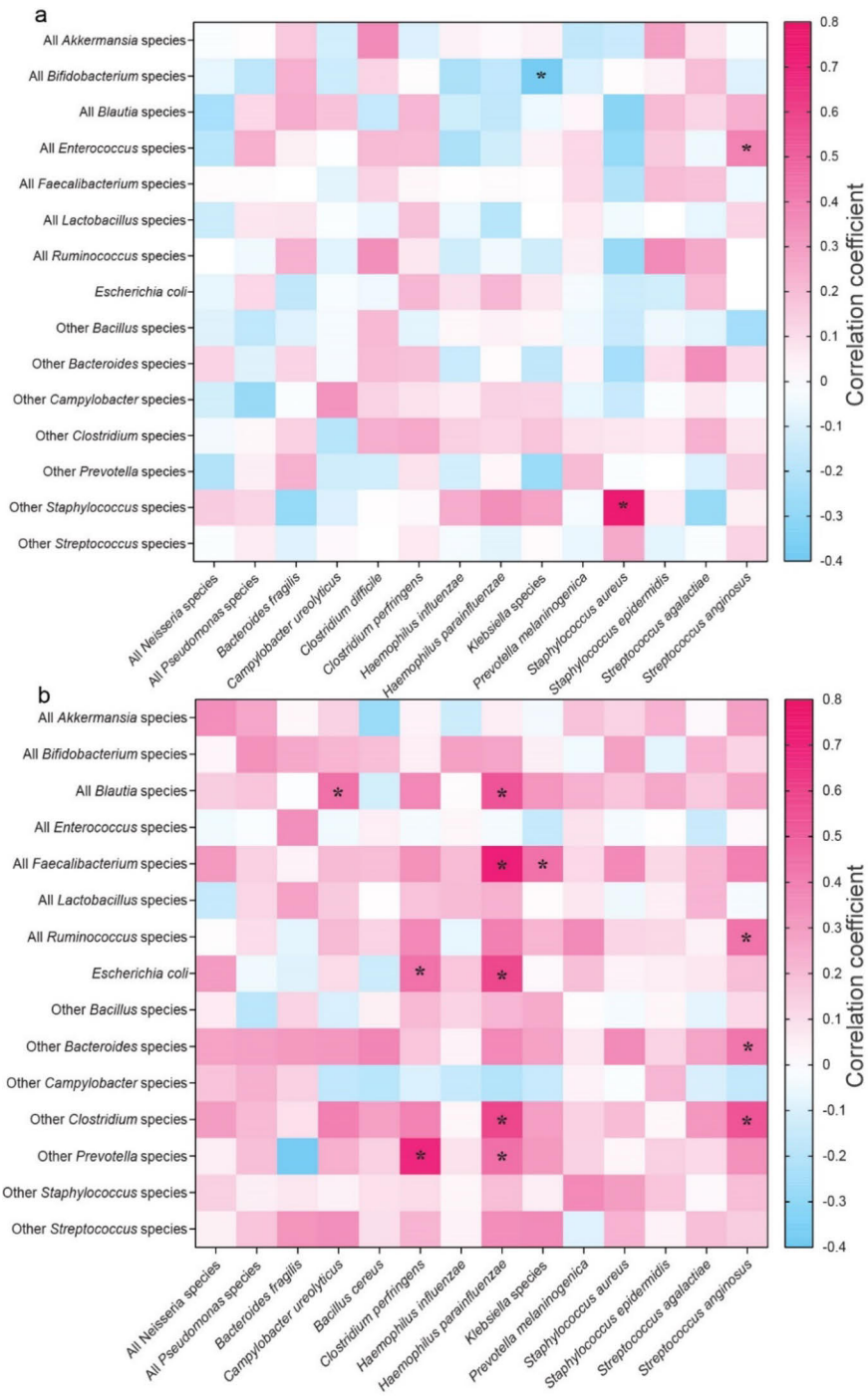
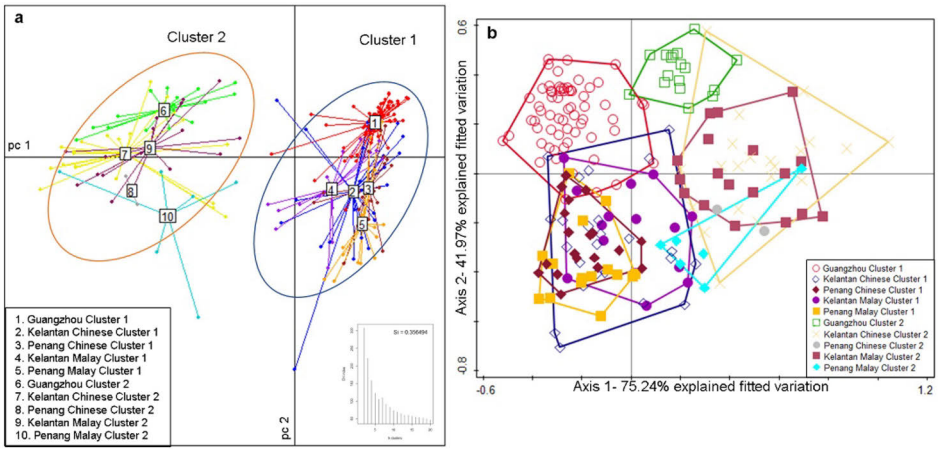
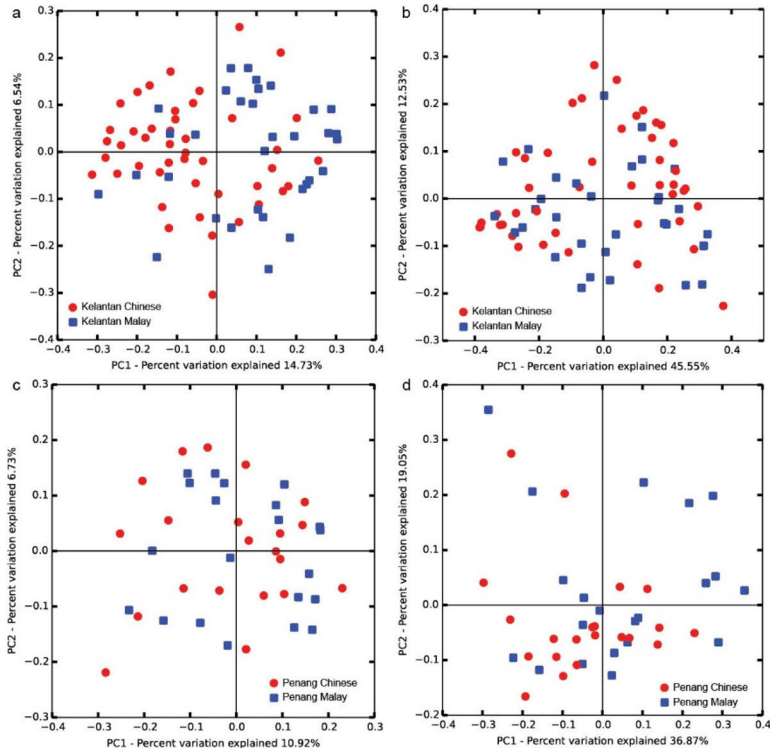
Appendices



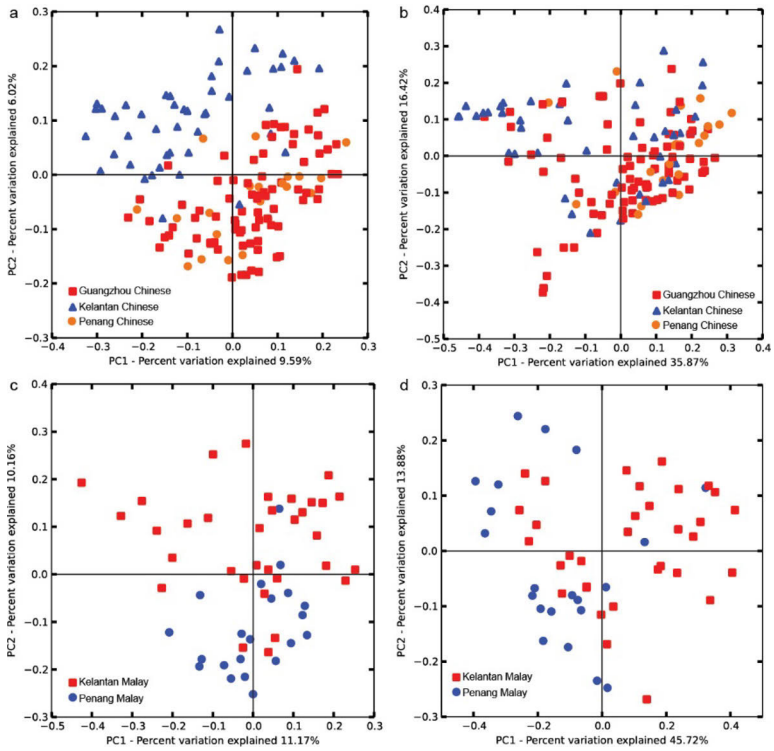
Appendix figure 1. Correlation heatmaps of commensal gut species (Y-axis) and potential pathogens (X-axis) in the fecal samples of children showing (a) before and (b) after weaning periods. *p ≤ 0.05. (Study I)



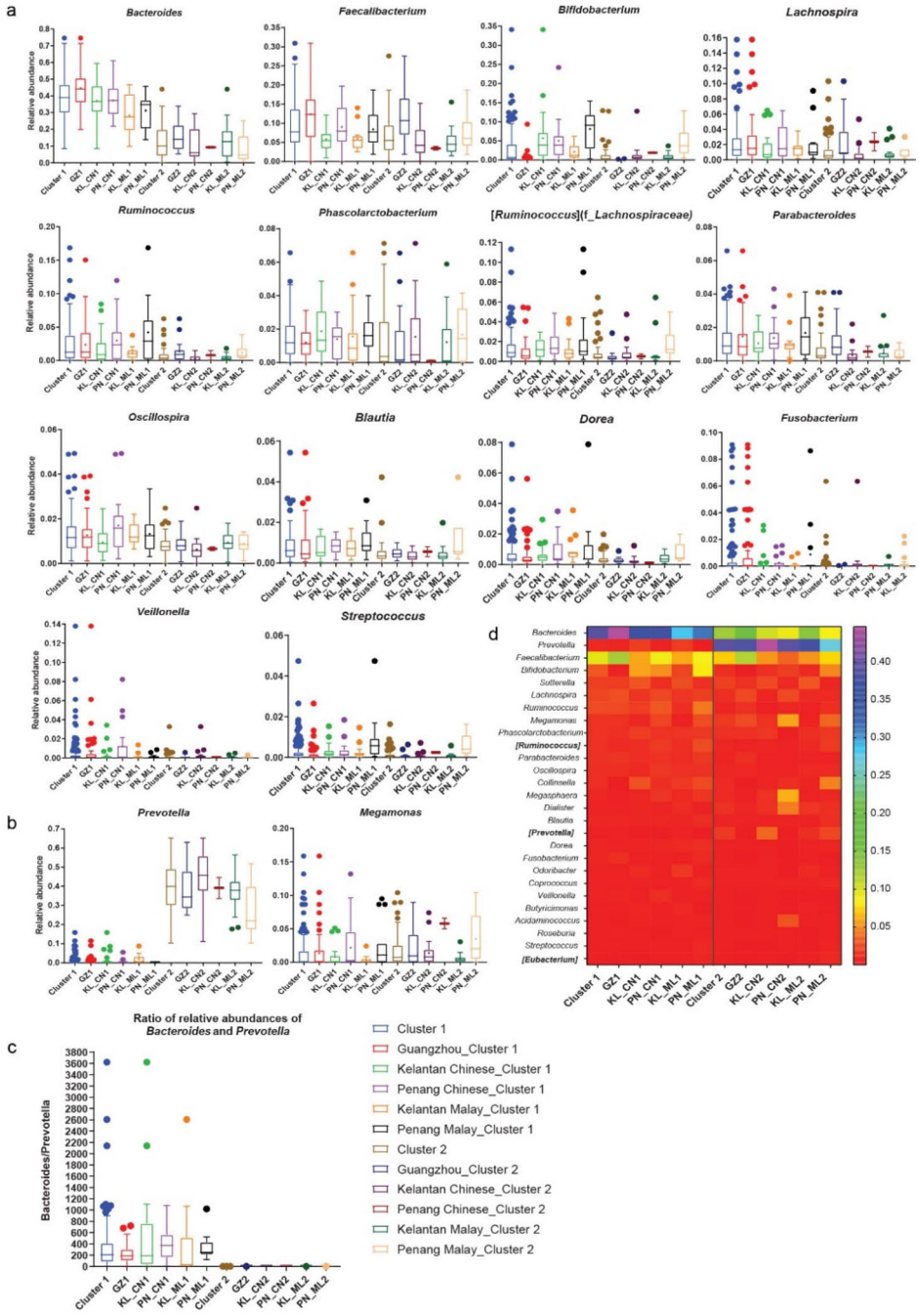
Appendix figure 2. (a) Principal coordinate analysis (PCoA) plot of PAM clustering using JSD and (b) distance-based redundancy analysis (db-RDA) plot using Bray-Curtis distance comparing two clusters, validated by the Calinski-Harabasz (CH) index, of fecal bacterial genera of two races living in three cities. (Study II)



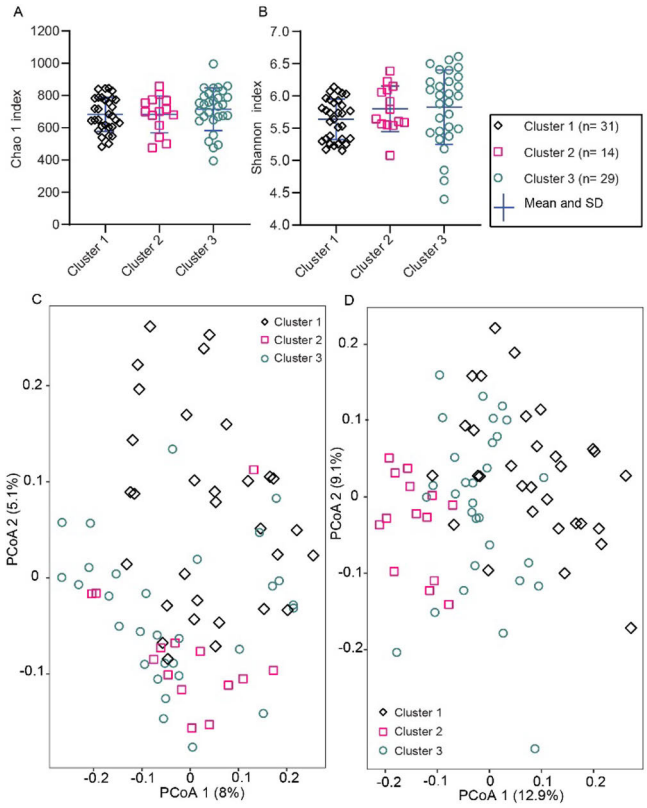
Appendix figure 3. Principal coordinate analysis (PCoA) plots of fecal microbiota beta diversity between two ethnicities in the same city (location) using (a,c) unweighted and (b,d) weighted UniFrac distances. (Study II)



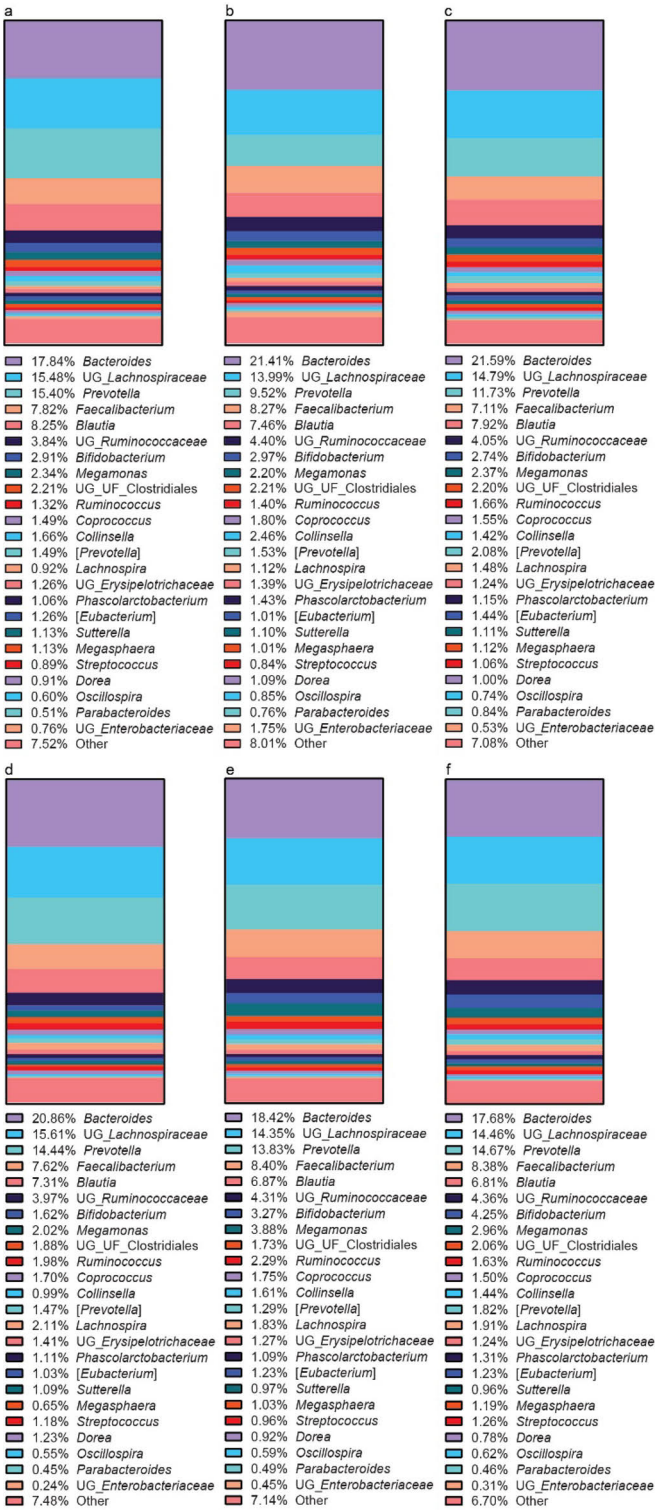
Appendix figure 4. Principal coordinate analysis (PCoA) plots of fecal microbiota beta diversity between cities in the same ethnicity using (a,c) unweighted and (b,d) weighted UniFrac distances. (Study II)



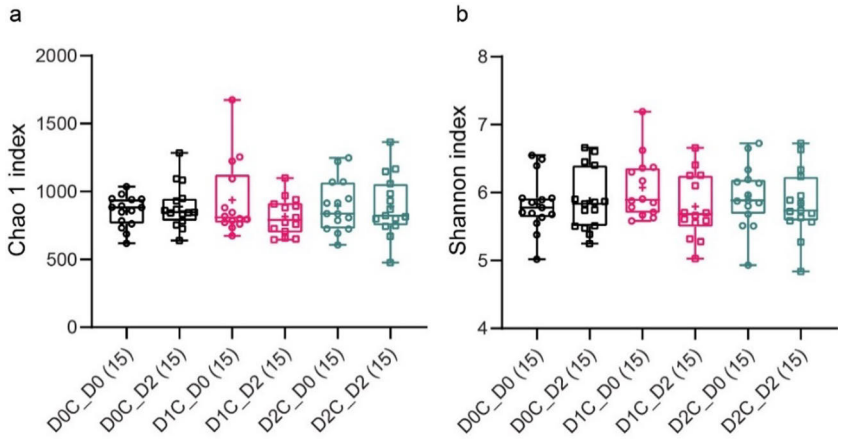
Appendix figure 5. Relative abundances of major bacterial genera (>1% of total OTUs) in fecal samples of two ethnicities living in three cities. (a) Cluster 1 (b) Cluster 2 (c) The ratio of *Bacteroides* and *Prevotella* abundances (d) Major bacterial profile. *p ≤ 0.05. KL= Kelantan city, PN= Penang city, GZ= Guangzhou city, CN= Southern Chinese, ML= Malay. The complete figures can be found in paper II. (Study II)



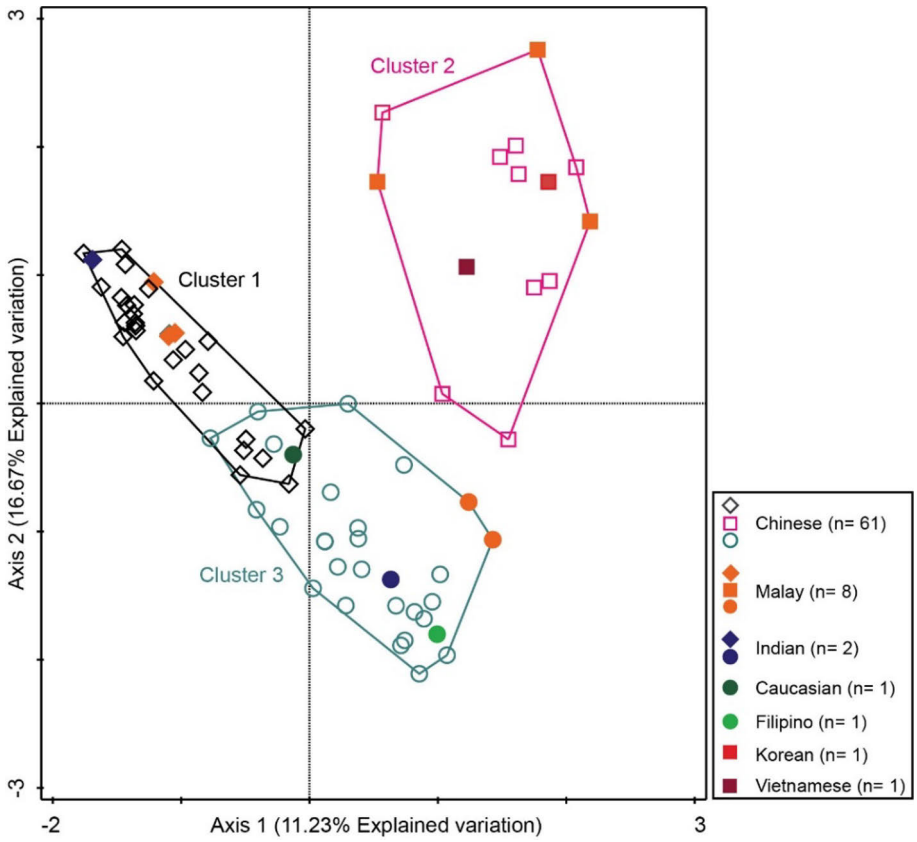
Appendix figure 6. (a) Chao1's and (b) Shannon's alpha diversity indices of three clusters at baseline. PCoA plots of beta diversity using (c) unweighted and (d) weighted UniFrac distances of three clusters at baseline. (Study III)



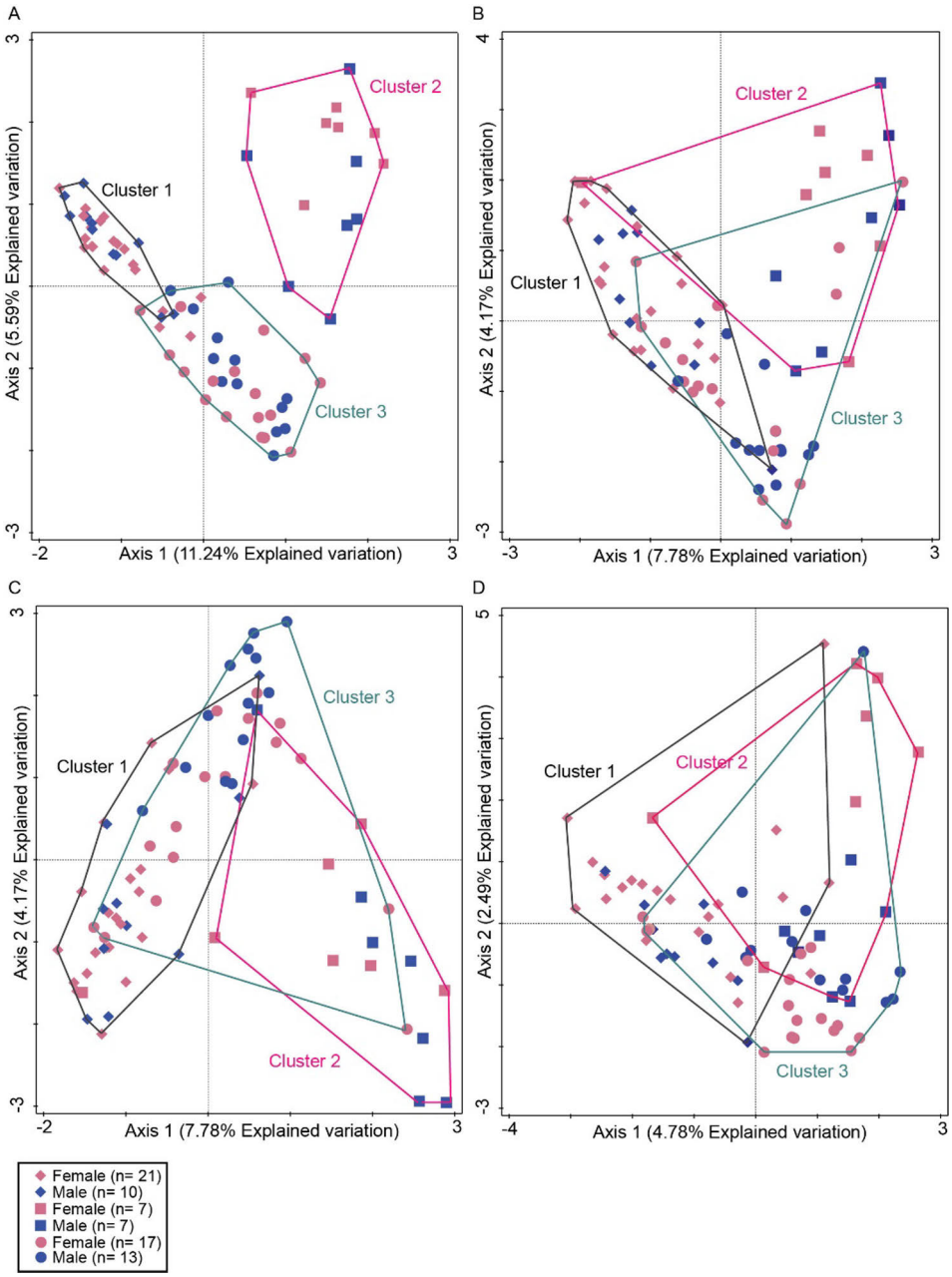
Appendix figure 7. Relative abundances of fecal bacterial genera (1% of total OTUs and above) of adults at (a-c) Day 0 and (d-f) Day 2 after three doses of spices intervention: (a,d) D0C, (b,e) D1C and (c,f) D2C. D0C= Meal with no spice intervention, D1C= Meal with low (6 g) mixed spices intervention, D2C= Meal with high (12g) mixed spices intervention, UG= Unknown genus, UF= Unknown family. (Study IV)



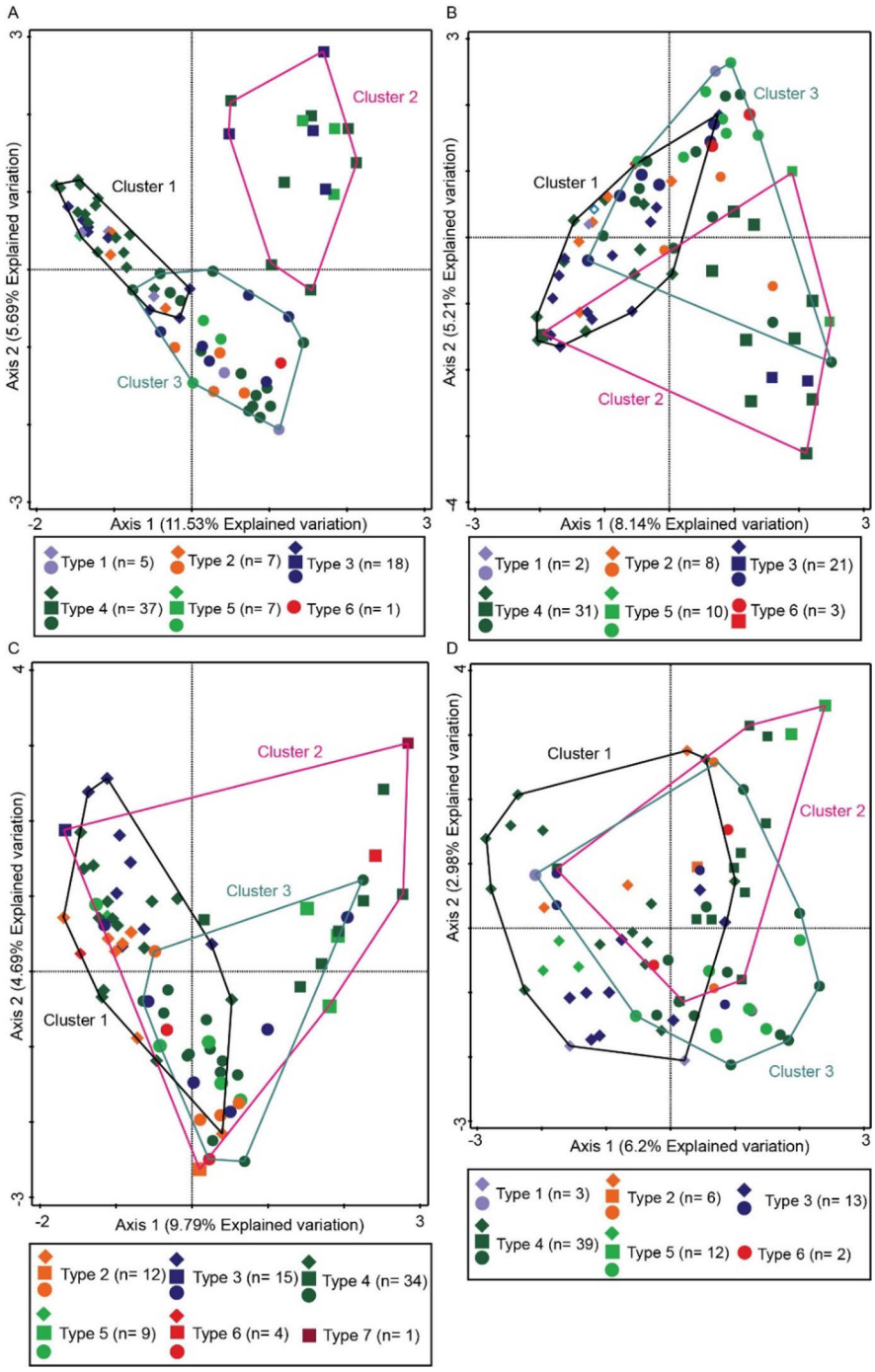
Appendix figure 8. (a) Chao1's and (b) Shannon's alpha diversity indices of three doses of spices intervention at Day 0 and Day 2. D0C= Meal with no spice intervention, D1C= Meal with low (6 g) mixed spices intervention, D2C= Meal with high (12g) mixed spices intervention. (Study IV)



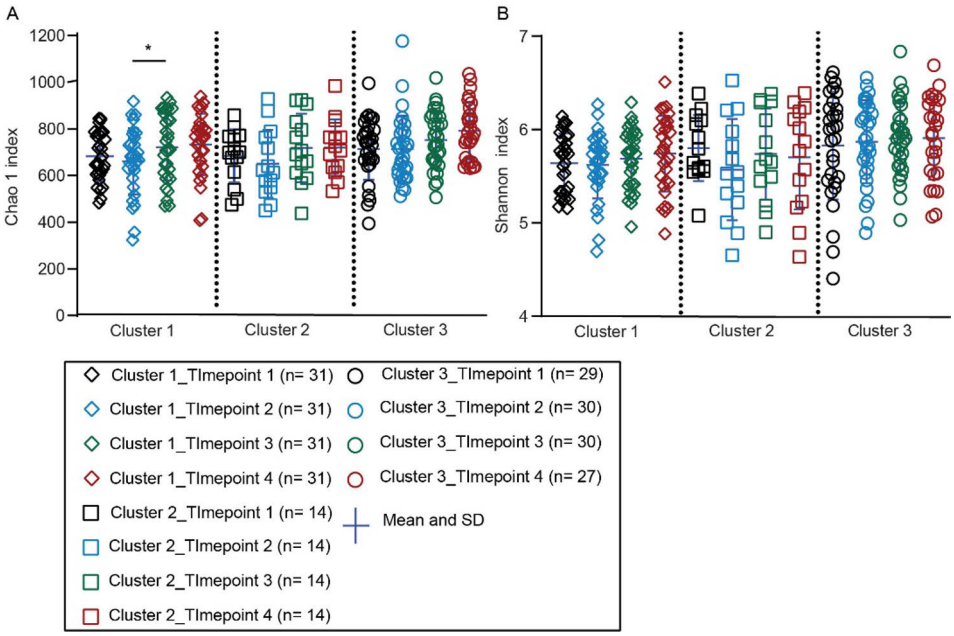
Appendix figure 9. Species biplot of distance-based redundancy analysis (db-RDA) using the square root Bray-Curtis distance matrix based on fecal microbiota composition in different ethnicities of three clusters at baseline. (Study III)



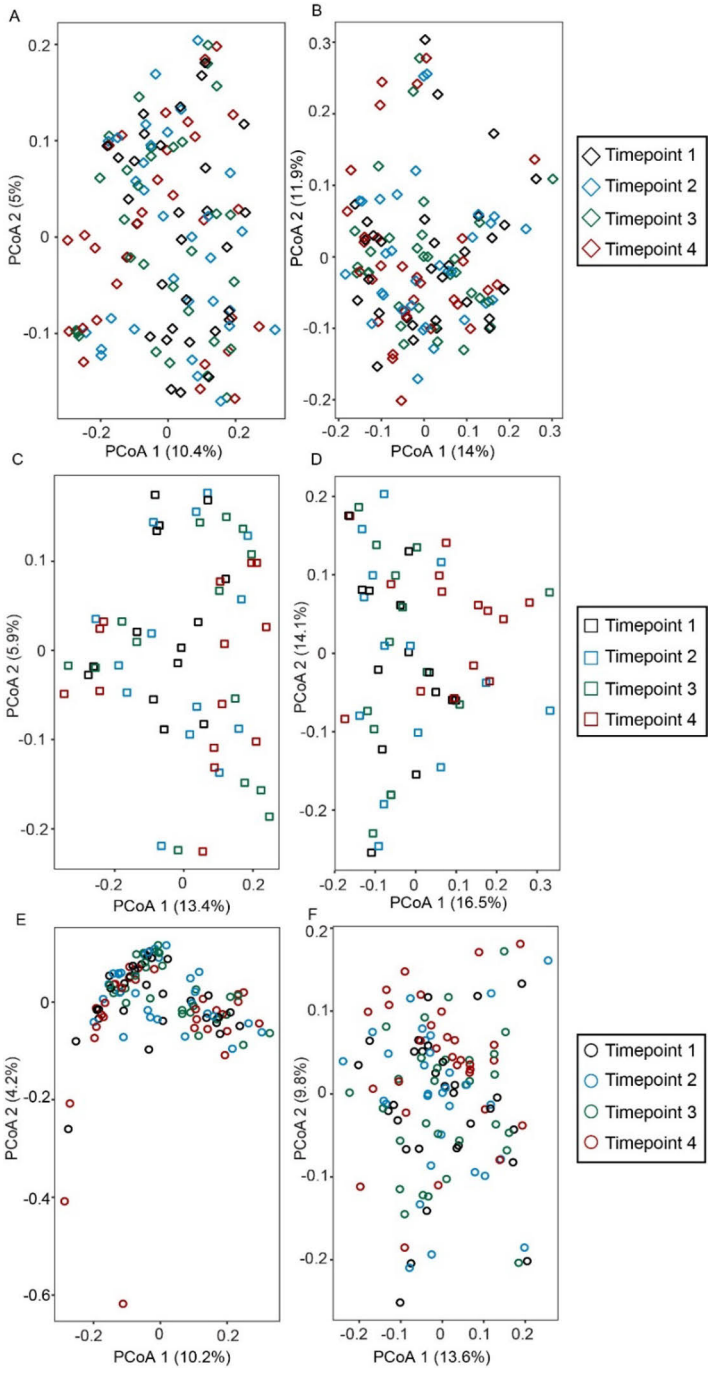
Appendix figure 10. Species biplots of distance-based redundancy analysis (db-RDA) using the square root Bray-Curtis distance matrix based on the composition of fecal microbiota in both males and females in three clusters at (A) baseline (B) Timepoint 2, (C) Timepoint 3, and (D) Timepoint 4. (Study III)



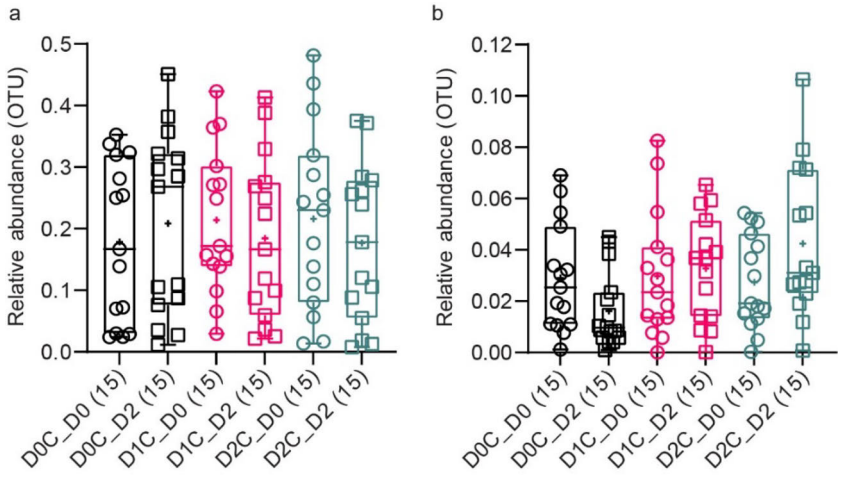
Appendix figure 11. Species biplots of distance-based redundancy analysis (db-RDA) using the square root Bray-Curtis distance matrix based on the composition of fecal microbiota in different Bristol stool scale types in three clusters at (A) baseline (B) Timepoint 2, (C) Timepoint 3, and (D) Timepoint 4. (Study III)



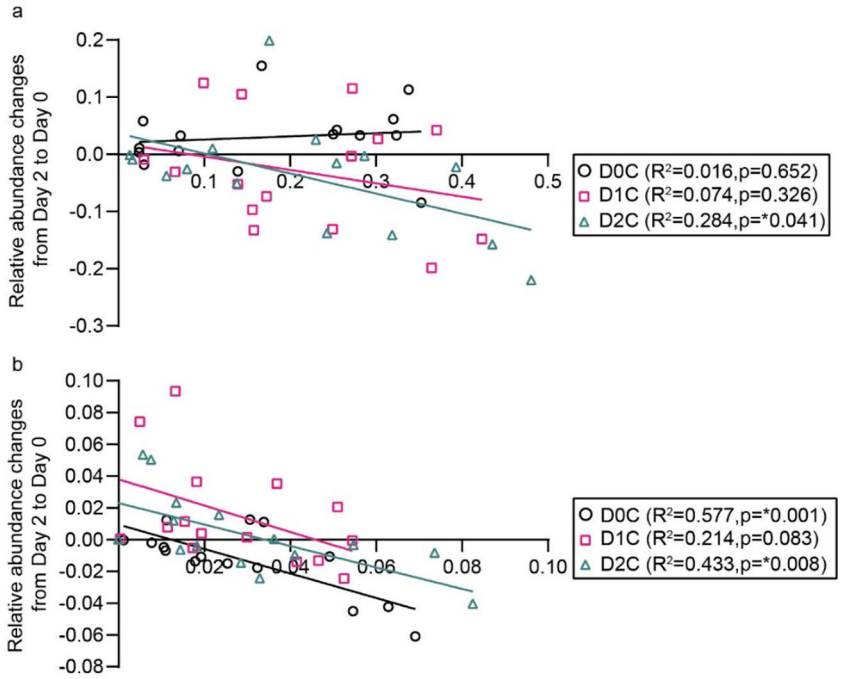
Appendix figure 12. (a) Chao1's and (b) Shannon's alpha diversity indices of three clusters across the time points. * $p \leq 0.05$. Timepoint 1= baseline; 14 days after washout, Timepoint 2= first 7 days after ingestion, Timepoint 3= second 7 days after ingestion, Timepoint 4= follow-up; 14 days after non-ingestion, SD= standard deviation. Numbers of samples in parenthesis. (Study III)



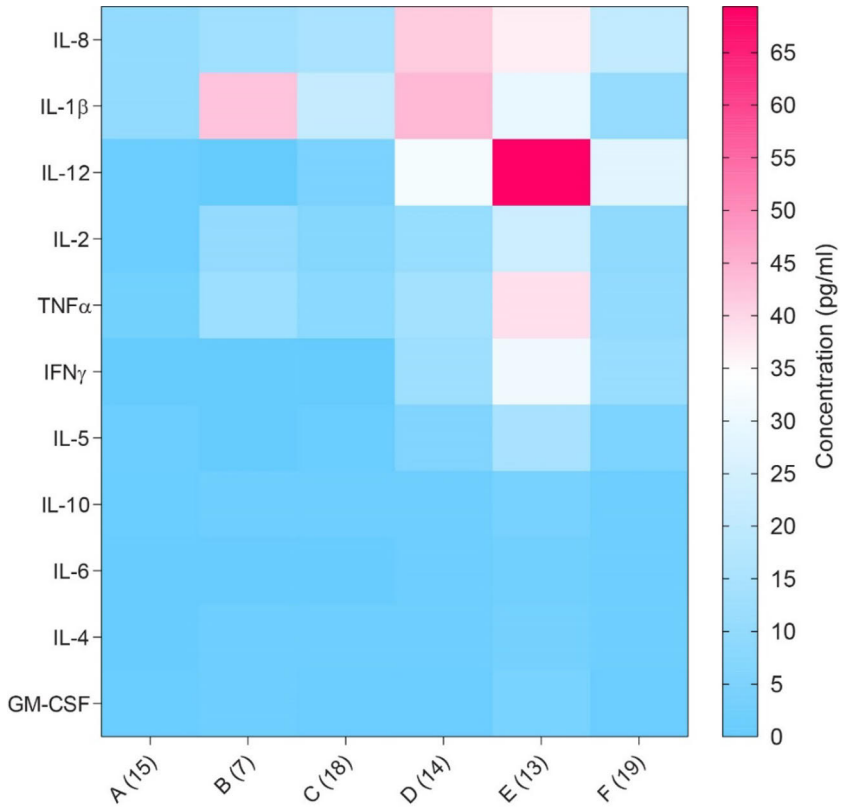
Appendix figure 13. (A, C, E) Unweighted and (B, D, F) weighted UniFrac PCoA of beta diversity comparing three clusters: (A, B) Cluster 1, (C, D) Cluster 2 and (E, F) Cluster 3 for all four time points. Timepoint 1= baseline; 14 days after washout, Timepoint 2= first 7 days after ingestion, Timepoint 3= second 7 days after ingestion, Timepoint 4= follow-up; 14 days after non-ingestion. (Study III)



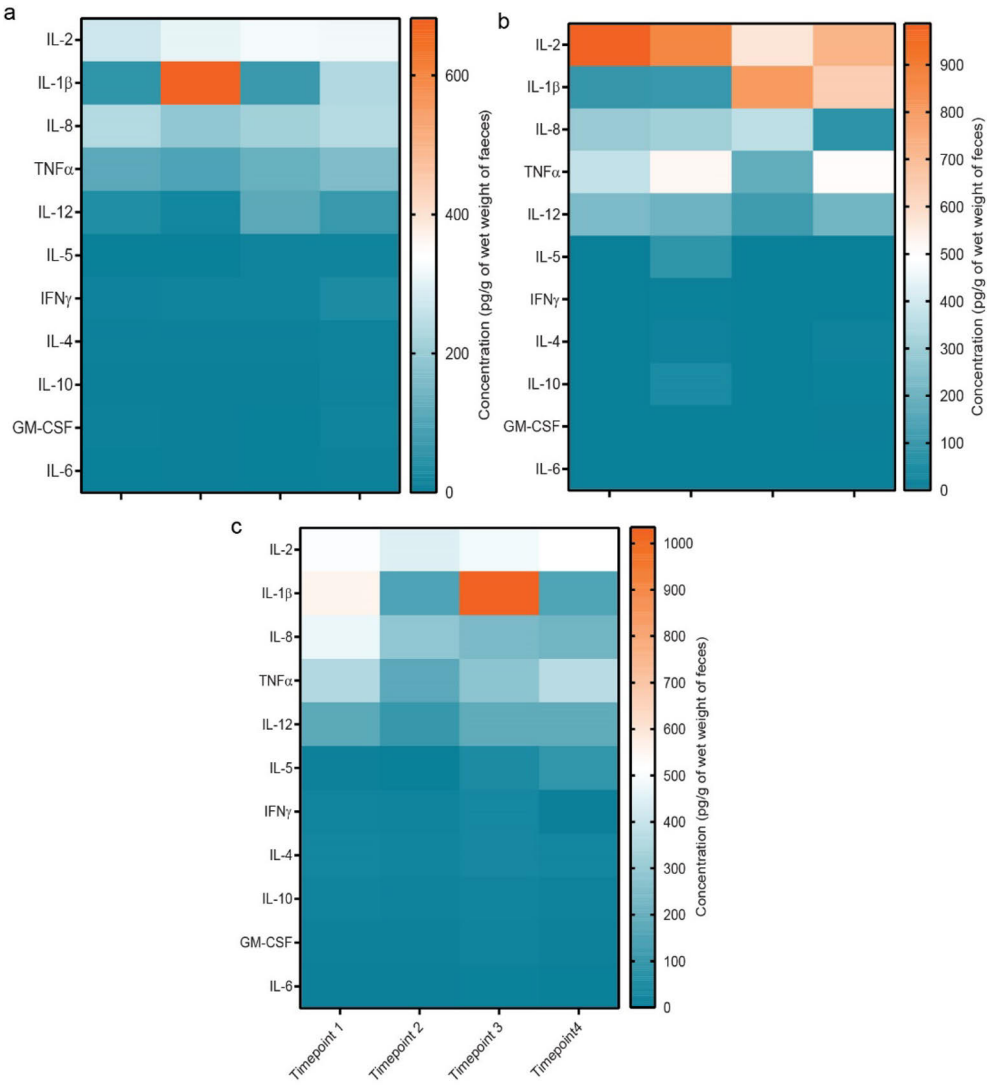
Appendix figure 14. Relative abundances of (a) *Bacteroides* and (b) *Bifidobacterium* at Day 0 and day 2 of three doses of mixed spices intervention. D0C= Meal with no spice intervention, D1C= Meal with low (6 g) mixed spices intervention, D2C= Meal with high (12g) mixed spices intervention. (Study IV)



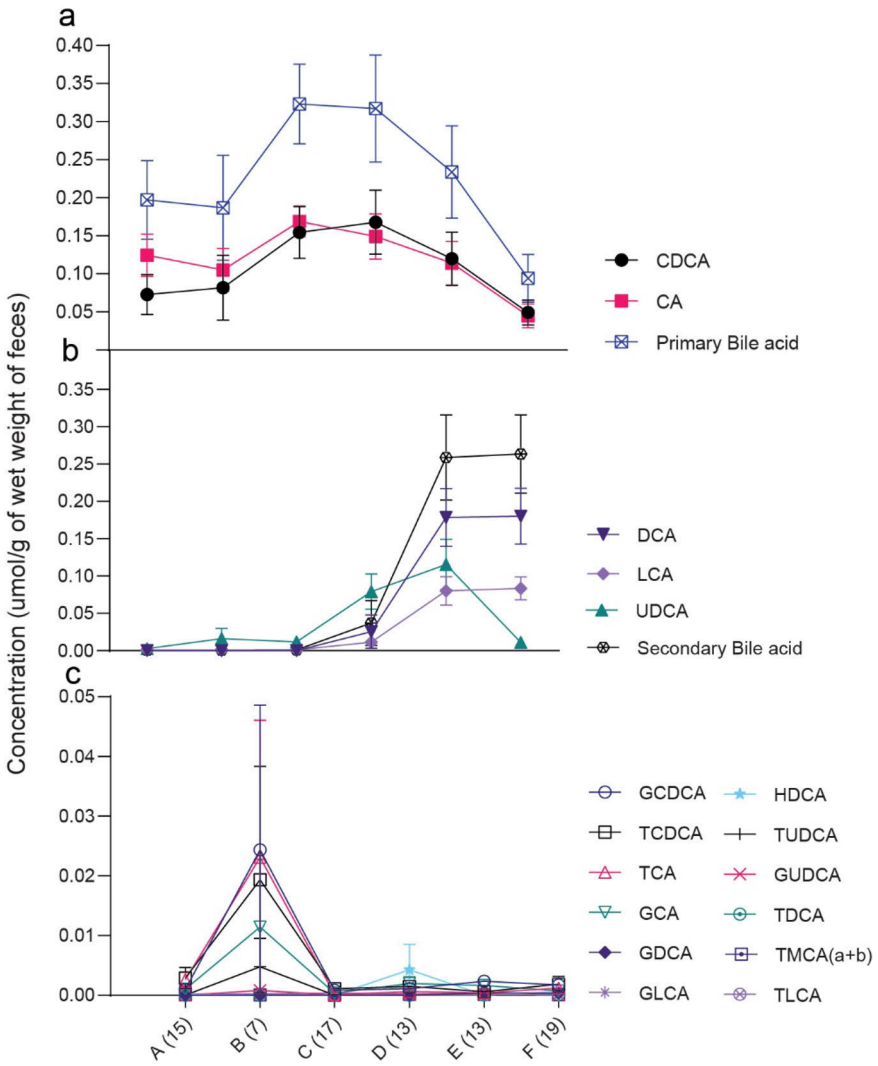
Appendix figure 15. Relationship (simple linear regression) between the changes in relative abundance (Y axis) of (a) *Bacteroides* and (b) *Bifidobacterium* and their relative abundance at Day 0 (X axis). D0C= Meal with no spice intervention, D1C= Meal with low (6 g) mixed spices intervention, D2C= Meal with high (12g) mixed spices intervention. (Study IV)



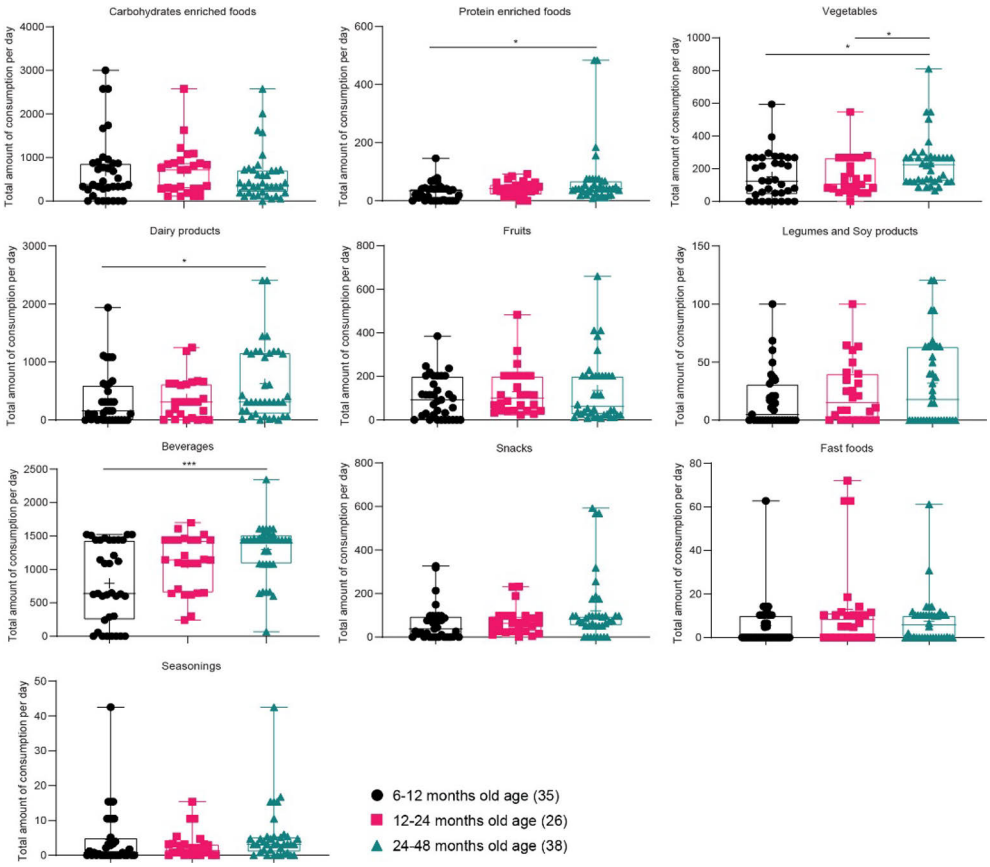
Appendix figure 16. Average concentrations of fecal water cytokines of children. No of samples in the parenthesis. A-D= less than 1 month, 1-3 months, 3-6 months and 6-12 months old age groups, respectively. IL= Interleukin, TNF= Tumour necrosis factor, IFN= Interferon, GM-CSF= Granulocyte-macrophage-stimulating factor. (Study I)



Appendix figure 17. Average concentrations of fecal water cytokines of adults (a) Cluster 1 (b) Cluster 2, and (c) Cluster 3. across the time points. Timepoint 1= baseline; 14 days after washout, Timepoint 2= first 7 days after ingestion, Timepoint 3= second 7 days after ingestion, Timepoint 4= follow-up; 14 days after cessation of ingestion. IL= Interleukin, TNF= Tumour necrosis factor, IFN= Interferon, GM-CSF= Granulocyte-macrophage-stimulating factor. (Study III)



Appendix figure 18. Average concentrations of fecal water free bile acids (a) primary bile acids (CDCA, CA) (b) secondary bile acids (DCA, LCA, UDCA) (c) 12 bile acids of children. Means and standard error of the mean (SEM) are presented. Numbers of samples in parentheses. A-F= less than 1 month, 1-3 months, 3-6 months, 6-12 months, 12-24 months, 24-48 months old age groups, respectively. CDCA= chenodeoxycholic acid, CA= colic acid; UDCA= ursodeoxycholic acid, DCA= deoxycholic acid, LCA= lithocholic acid; GCDCA= glycochenodeoxycholic acid, TCDCA= taurochenodeoxycholic acid, TCA= taurocholic acid, GCA= glycocholic acid, GDCA= glycodeoxycholic acid, GLCA= glycolithocholic acid, HDCA= hyodeoxycholic acid, TUDCA= tauroursodeoxycholic acid, GUDCA= glycoursodeoxycholic acid, TDCA= taurodeoxycholic acid, TMCA [a+b]= alpha- and beta-tauromuricholic acid, and TLCA= taurolithocholic acid. (Study I)



Appendix figure 19. Total amount of dietary consumption (g) per day of children. *p ≤ 0.05. Number of samples in the parentheses. (Study I)

Appendix table 1. Results of the pairwise comparison statistical test of the relative abundances of major genera comparing the same city and ethnicity, comparing the different ethnicities in the same city, and comparing the same ethnicities in the different cities. (Study II)

Comparisons	Pairs	*Bacteroides*	*Prevotella*	*Faecalibacterium*	*Bifidobacterium*	*Sutterella*	*Lachnospira*	*Ruminococcus*	*[Ruminococcus]*	*Parabacteroides*	*Oscillospira*
the same city and ethnicity	GZ1 vs GZ2	<0.0001	<0.0001								
	KLCN1 vs KLCN2	<0.0001	<0.0001							0.0347	
	PNCN1 vs PNCN2										
	KLML1 vs KLML2		0.0052								
	PNML1 vs PNML2		0.0022								
the different ethnicities in the same city	KLCN1 vs KLML2	0.0005	<0.0001								
	KLML1 vs KLCN2		0.0002								0.0121
	PNCN1 vs PNML2	0.0179	0.0024								
	PNML1 vs PNCN2										
	KLCN1 vs KLML1										
	PNCN1 vs PNML1										
	KLCN2 vs KLML2										
	PNCN2 vs PNML2										
the same ethnicities in the different cities	GZ1 vs KLCN2	<0.0001	<0.0001	0.0003			0.0018	<0.0001		0.0038	0.0051
	GZ1 vs PNCN2										
	KLCN1 vs GZ2	0.0041	<0.0001		<0.0001						
	KLCN1 vs PNCN2										
	PNCN1 vs GZ2	0.0084	<0.0001		<0.0001				0.0041		
	PNCN1 vs KLCN2	<0.0001	<0.0001					0.0033		0.0041	0.0042
	KLML1 vs PNML2										
	PNML1 vs KLML2		<0.0001		0.0147	0.0341		0.0271			
	GZ1 vs KLCN1			0.0026	<0.0001						
	GZ1 vs PNCN1				<0.0001						
	KLCN1 vs PNCN1										
	KLML1 vs PNML1										
	GZ2 vs KLCN2			0.0366	0.0055						
	GZ2 vs PNCN2										
	KLCN2 vs PNCN2										
	KLML2 vs PNML2					0.0079					

p values were calculated from the statistical test of Kruskal Wallis, and Dunn's multiple comparisons test based on the relative abundances of major genera (> 1% of total bacterial genera). The significantly different bacteria compared to the same ethnicities in the different cities are described and p values cut-off is < 0.05. Non-significant differences bacteria are not shown. KL= Kelantan, PN= Penang, GZ= Guangzhou, CN= Southern Chinese, M= Malay, 1= Cluster 1, 2=Cluster 2.

Appendix table 1: Continued

Comparisons	Pairs	*Collinsella*	*Megasphaera*	*[Prevotella]*	*Fusobacterium*	*Odoribacter*	*Butyricimonas*	*Streptococcus*	*[Eubacterium]*	*Dorea*
the same city and ethnicity	GZ1 vs GZ2									
	KLCN1 vs KLCN2							0.0302		
	PNCN1 vs PNCN2									
	KLML1 vs KLML2									
	PNML1 vs PNML2									
the different ethnicities in the same city	KLCN1 vs KLML2									
	KLML1 vs KLCN2					0.0001				0.0005
	PNCN1 vs PNML2									
	PNML1 vs PNCN2									
	KLCN1 vs KLML1									
	PNCN1 vs PNML1									
	KLCN2 vs KLML2									
	PNCN2 vs PNML2									
the same ethnicities in the different cities	GZ1 vs KLCN2	0.0002			0.0263					
	GZ1 vs PNCN2									
	KLCN1 vs GZ2	0.0001		<0.0001						
	KLCN1 vs PNCN2									
	PNCN1 vs GZ2	0.014								
	PNCN1 vs KLCN2									
	KLML1 vs PNML2									
	PNML1 vs KLML2						0.0096	0.0023	0.0004	
	GZ1 vs KLCN1	<0.0001		<0.0001	0.0031					
	GZ1 vs PNCN1	<0.0001			0.0452				0.0211	
	KLCN1 vs PNCN1									
	KLML1 vs PNML1					0.0061	0.0095			
	GZ2 vs KLCN2		0.0447	0.0196						
	GZ2 vs PNCN2									
	KLCN2 vs PNCN2									
	KLML2 vs PNML2							0.0487		

p values were calculated from the statistical test of Kruskal Wallis, and Dunn's multiple comparisons test based on the relative abundances of major genera (> 1% of total bacterial genera). The significantly different bacteria compared to the same ethnicities in the different cities are described and p values cut-off is < 0.05. Non-significant differences bacteria are not shown. KL= Kelantan, PN= Penang, GZ= Guangzhou, CN= Southern Chinese, M= Malay, 1= Cluster 1, 2=Cluster 2.

Appendix table 2. Relative abundance of OTUs of major (>1% of total OTUs) fecal bacterial genera at baseline. (Study III)

Bacteria	Cluster 1	Cluster 2	Cluster 3
Bacteroides	0.305	0.082	0.117
Blautia	0.116	0.116	0.159
Lachnospiraceae	0.142	0.110	0.116
Faecalibacterium	0.094	0.101	0.122
Ruminococcaceae	0.033	0.061	0.084
Prevotella	0.002	0.213	0.011
Coprococcus	0.029	0.041	0.059
Bifidobacterium	0.035	0.031	0.049
[Ruminococcus]	0.029	0.013	0.027
Ruminococcus	0.008	0.008	0.041
Dorea	0.019	0.010	0.013
Collinsella	0.009	0.017	0.016
Clostridiales	0.009	0.012	0.017
Streptococcus	0.011	0.012	0.013
Lachnospira	0.016	0.007	0.010
Megamonas	0.015	0.019	0.004
[Eubacterium]	0.002	0.010	0.020
Megasphaera	0.014	0.008	0.009
Oscillospira	0.008	0.007	0.009
Enterobacteriaceae	0.006	0.014	0.004
Parabacteroides	0.009	0.006	0.005
Phascolarctobacterium	0.007	0.007	0.005
Clostridiales;Other;Other	0.006	0.005	0.007
Erysipelotrichaceae	0.006	0.008	0.005
Lactobacillus	0.003	0.008	0.006
Sutterella	0.009	0.003	0.003

Cluster 1, n= 31; Cluster 2, n= 14; Cluster 3, n= 30. OTU= operational taxonomical unit.

Appendix table 3. Results of the two-way ANOVA and Bonferroni's multiple pairwise comparison tests of cytokines in the fecal water of children. (Study I)

Two-way ANOVA (p values) = ****<0.0001**

Pairs	IL-8	IL-1β	IL-12	IL-2	TNFα	IFNɣ	IL-4, -5, -6, -10 and GM-CSF
A vs. B	>1	***0.0101**	>1	>1	>1	>1	>1
A vs. C	>1	>1	>1	>1	>1	>1	>1
A vs. D	***0.0007**	***0.0002**	**0.0012**	>1	>1	>1	>1
A vs. E	*0.0118	0.20	****<0.0001**	0.09	***0.0001**	**0.0015**	>1
A vs. F	>1	>1	**0.0038**	>1	>1	>1	>1
B vs. C	>1	0.37	>1	>1	>1	>1	>1
B vs. D	0.05	>1	*0.0159	>1	>1	>1	>1
B vs. E	0.26	>1	****<0.0001**	>1	0.11	*0.0247	>1
B vs. F	>1	**0.0097**	*0.0438	>1	>1	>1	>1
C vs. D	**0.0058**	*0.0396	**0.0042**	>1	>1	>1	>1
C vs. E	0.07	>1	****<0.0001**	0.57	**0.0012**	***0.0008**	>1
C vs. F	>1	>1	*0.0131	>1	>1	>1	>1
D vs. E	>1	>1	****<0.0001**	>1	*0.0312	0.34	>1
D vs. F	0.07	***0.0001**	>1	>1	>1	>1	>1
E vs. F	0.53	0.20	****<0.0001**	>1	**0.0021**	0.14	>1

p values derived from ordinary two-way ANOVA test based on the concentration (pg/ml) of cytokines and adjusted p values from Bonferroni's multiple pairwise comparison test are described. The cytokines which were significantly different between pairs of time points are represented as **** $p < 0.0001$, *** $p \geq 0.0001$ - < 0.001, ** $p \geq 0.001$ - < 0.01, * $p \geq 0.01$- 0.05. A = less than 1-month old (n= 15), B = 1-3 months old (n= 7), C = 3-6 months old (n= 18), D = 6-12 months (n= 14), E = 12-24 months old (n= 13), F = 24-48 months old (n= 19).

Appendix table 4. Average concentration of fecal water cytokines (pg/g of wet weight of faeces) in all clusters throughout the time points (a) Timepoint 1 (b) Cluster 1 (c) Cluster 2 (d) Cluster 3. (Study III)

Cytokines	(a) Timepoint 1 Mean ± SD (pg/g of wet weight of faeces)				
	Cluster 1	Cluster 2	Cluster 3	Cluster 2/Cluster1	Cluster 3/Cluster 1
IL-2	272.48 ± 532.25	987.23 ± 678.55	506.81 ± 724.33	3.62	1.86
IL-1β	55.63 ± 173.79	88.71 ± 225.12	558.38 ± 1598.87	1.59	10.04
IL-8	235.61 ± 721.5	289.01 ± 498.38	475.25 ± 1405.2	1.23	2.02
TNFα	113.45 ± 355.39	372.15 ± 651.99	355.54 ± 670.01	3.28	3.13
IL-12	37.03 ± 144.47	229.25 ± 348.43	173.56 ± 336.99	6.19	4.69
IL-5	0 ± 0	0 ± 0	7.36 ± 27.05	ND	ND
IFNɣ	7.11 ± 28.91	0 ± 0	18.33 ± 47.32	0	2.58
IL-4	3.4 ± 18.62	0 ± 0	25.09 ± 44.41	0	7.38
IL-10	1.58 ± 8.65	0 ± 0	15.73 ± 24.83	0	9.96
GM-CSF	4.71 ± 11.89	0 ± 0	6.2 ± 16.81	0	1.32
IL-6	0 ± 0	0 ± 0	0.92 ± 4.89	ND	ND

(b) Cluster 1 Mean ± SD (pg/g of wet weight of faeces)				
Cytokines	Timepoint 1	Timepoint 2	Timepoint 3	Timepoint 4
IL-2	272.48 ± 532.25	304.37 ± 532.5	321.47 ± 580.95	316.82 ± 524.5
IL-1β	55.63 ± 173.79	681.93 ± 3288.75	68.16 ± 119.66	233.32 ± 743.26
IL-8	235.61 ± 721.5	190.78 ± 308.78	216.02 ± 298.02	239.07 ± 403.48
TNFα	113.45 ± 355.39	97.6 ± 319.96	132.42 ± 374.41	165.15 ± 581.52
IL-12	37.03 ± 144.47	15.91 ± 78.07	116 ± 247.81	66.83 ± 209.36
IL-5	0.00 ± 0.00	0.00 ± 0.00	8.61 ± 24.41	9.53 ± 20.32
IFNɣ	7.11 ± 28.91	8.93 ± 31.83	9.08 ± 28.08	31.52 ± 85.1
IL-4	3.4 ± 18.62	3.95 ± 18.83	3.55 ± 15.04	6.42 ± 32.78
IL-10	1.58 ± 8.65	2.27 ± 12.45	0.82 ± 4.46	5.43 ± 23.37
GM-CSF	4.71 ± 11.89	0.79 ± 4.34	0.00 ± 0.00	8.86 ± 32.26
IL-6	0.00 ± 0.00	0.35 ± 1.93	0.00 ± 0.00	5.02 ± 27.5

(c) Cluster 2 Mean ± SD (pg/g of wet weight of faeces)				
Cytokines	Timepoint 1	Timepoint 2	Timepoint 3	Timepoint 4
IL-2	987.23 ± 678.55	866.96 ± 808.32	570.43 ± 695.64	722.13 ± 788.73
IL-1β	88.71 ± 225.12	95.4 ± 237.4	800.67 ± 2991.99	641.03 ± 2099.72
IL-8	289.01 ± 498.38	308.73 ± 368.57	360.52 ± 1192.21	76.74 ± 138.2
TNFα	372.15 ± 651.99	522.31 ± 776.01	180.78 ± 459.91	503.48 ± 772.05
IL-12	229.25 ± 348.43	201.42 ± 404.27	108.84 ± 217.94	212.92 ± 359.75
IL-5	0.00 ± 0.00	81.08 ± 303.39	0.00 ± 0.00	0.00 ± 0.00
IFNɣ	0.00 ± 0.00	3.92 ± 14.67	0.00 ± 0.00	0.00 ± 0.00
IL-4	0.00 ± 0.00	10.93 ± 38.33	0.00 ± 0.00	7.71 ± 28.86
IL-10	0.00 ± 0.00	43.41 ± 162.41	0.00 ± 0.00	4.47 ± 16.73
GM-CSF	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	3.07 ± 11.49
IL-6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

(d) Cluster 3 Mean ± SD (pg/g of wet weight of faeces)				
Cytokines	Timepoint 1	Timepoint 2	Timepoint 3	Timepoint 4
IL-2	506.81 ± 724.33	446.55 ± 641.26	483.66 ± 672.13	520.52 ± 760.58
IL-1β	558.38 ± 1598.87	146.49 ± 291.52	1034.57 ± 3263.82	148.44 ± 310.56
IL-8	475.25 ± 1405.2	284.84 ± 528.72	237.48 ± 568.56	219.51 ± 446.5
TNFα	355.54 ± 670.01	171.97 ± 445.47	278 ± 498.76	367.3 ± 676.38
IL-12	173.56 ± 336.99	99.04 ± 251.61	181.93 ± 284.1	180.95 ± 380.17
IL-5	7.36 ± 27.05	0.00 ± 0.00	46.8 ± 241.06	88.75 ± 303.61
IFNɣ	18.33 ± 47.32	13.46 ± 52.28	32.41 ± 83.92	3.45 ± 18.26
IL-4	25.09 ± 44.41	16.23 ± 35.43	31.53 ± 64.71	25.46 ± 49.27
IL-10	15.73 ± 24.83	8.33 ± 24.06	17.48 ± 35.36	9.56 ± 24.81
GM-CSF	6.2 ± 16.81	6.7 ± 17.77	15.01 ± 30.27	7.66 ± 22.6
IL-6	0.92 ± 4.89	2.16 ± 11.24	4.11 ± 15.11	0.00 ± 0.00

ND= Not Determined. IL= Interleukin, TNF= Tumour necrosis factor, IFN= Interferon, GM-CSF= Granulocyte-macrophage-stimulating factor, SD= standard deviation.

Appendix Table 5. Frequency of dietary consumption per week at baseline (Mean ± SD). (Study III)

Foods	Cluster 1	Cluster 2	Cluster 3
Carbohydrate-rich foods	30.72 ± 12.19	18.38 ± 7.60	36.16 ± 11.38
Protein-rich foods	35.94 ± 13.68	23.98 ± 14.53	41.06 ± 18.04
Vegetables	5.22 ± 3.03	11.00 ± 4.57	5.53 ± 2.57
Fruits	3.61 ± 4.49	2.80 ± 2.14	4.28 ± 2.72
Nuts	4.11 ± 3.81	2.70 ± 3.71	4.13 ± 3.46
Beverages	16.11 ± 6.82	9.25 ± 13.02	15.06 ± 6.11
the highest frequency of consumption (no. of food types)	1	1	4
the lowest frequency of consumption (no. of food types)	1	5	0

SD= standard deviation.

Appendix table 6. Mean daily intake of energy and nutrients during the run-in periods before each intervention session and the statistical result. (Study IV)

Measurement	Mean ± SD			p value (one-way ANOVA; Mixed-effects analysis)
	Dose 0 Control (D0C) (n=14)	Dose 1 Curry (D1C) (n=15)	Dose 2 Curry (D2C) (n=15)	
Energy (kcal)	1763.600 ± 377.666	1572.576 ± 429.014	1560.602 ± 406.569	0.1384
Carbohydrates (g)	189.385 ± 38.211	179.072 ± 51.176	183.486 ± 78.424	0.8262
Protein (g)	88.338 ± 27.345	78.561 ± 19.003	76.277 ± 20.264	0.1771
Total fats (g)	67.668 ± 19.673	57.709 ± 20.035	59.500 ± 20.667	0.2109
Saturated fats (g)	27.000 ± 9.586	23.832 ± 8.562	24.982 ± 7.659	0.5338
Fibre (g)	15.726 ± 6.151	13.969 ± 6.380	15.566 ± 7.358	0.417
Cholesterol (mg)	364.247 ± 165.379	301.156 ± 119.466	333.020 ± 162.609	0.2956
Sodium (mg)	3191.363 ± 766.049	2910.272 ± 861.957	3163.564 ± 1007.169	0.5686

P values were calculated from the statistical test of one-way ANOVA based on the consumption of each nutrient. D0C = Meal with no spices intervention, D1C =Meal with low (6g) mixed spices intervention, D2C = Meal with high (12g) mixed spices intervention, D0 = Day 0 (Baseline), D2 = Day 2. n= 15 each intervention.

Appendix Text 1: Weaning Food Frequency Questionnaire (I)

No.	Food Items	Frequency (times)			Portion Size
Complex Carbohydrates		**Per month**	**Per Week**	**PerDay**	
1.	Bread (White, wholemeal,..)				slice/pcs
2.	Cereal (Instant, manufactured, Babycereal,..)				tbsp/small bowl
3.	Chapatti				pcs/slice
4.	Dosai				pcs/slice
5.	Millet				tbsp/pcs
6.	Oatmeal				tbsp/small bowl
7.	Noodles (Yellow noodles, Ban Mian,Meehoon, Vermicelli, Pasta, Spaghetti, Macaroni,..)				tbsp/small bowl
8.	Rice (Porridge, Rice congee, NasiLemak, Nasi Biryani, Fried Rice, Butter Rice,..)				tbsp/small bowl
9.	Pizza				pcs/slice
10.	Parata				pcs/slice
11.	Quinoa				tbsp/small bowl
12.	Others:				

No.	Food Items	Frequency (times)			Portion Size
Vegetables		**Per month**	**Per Week**	**PerDay**	
13.	Flowerly (Cauliflower, Brococoli,Kai Lan, Bean sprouts, Bottle gourd,..)				tbsp/stalks
14.	Leafy (Spinach, Cabbage, Chinesecabbage, Water grass,..)				tbsp/pcs
15.	Root (Carrot, Radish, Sweet Potato,..)				tbsp/pcs
16.	Stem (Asparagus, Celery, ..)				tbsp/stalks
17.	Fruity (Cucumber, eggplant, tomato,pumpkin,..)				tbsp/pcs
18.	Tubers (Potato, Yam,..)				tbsp/pcs
19.	Seeds (Corns, Long beans, Peas,Green peas,..)				tbsp/seeds
20.	Bulbs (Spring onions, Garlic, Ginger,..)				tbsp
21.	Mushrooms				tbsp/pcs
22.	Others:				

No.	Food Items	Frequency (times)			Portion Size
Legumes and Soys		**Per month**	**Per Week**	**PerDay**	
23.	Legumes (Chickenpeas, Lentils,...)				tbsp/pcs/slice
24.	Beans (Red beans, Green beans,..)				tbsp/pcs/slice
25.	Soys (Tofu, Beancurd, Mock meats,Tau kwa,..)				tbsp/pcs/slice
Fruits					
26.	Fleshy (Apple, Banana, Pear, Papaya, Pineapple, Watermelon,Guava, Dragon fruits,..)				tbsp/pcs/slice
27.	Seeded (Mango, Avocado, Lychee,Plum, Prune,..)				tbsp/pcs/slice
28.	Berries (Grapes, Strawberry, Cherry,Blueberries, Cranberries,..)				tbsp/pcs
29.	Citrus (Orange, Lime, Grapefruit,Lemon, Mandarin,,..)				tbsp/pcs
30.	Others:				

No.	Food Items	Frequency (times)			Portion Size
Meats		**Per month**	**Per Week**	**PerDay**	
31.	Chicken				tbsp/pcs/slice
32.	Pork				tbsp/pcs/slice
33.	Beef				tbsp/pcs/slice
34.	Mutton				tbsp/pcs/slice
35.	Fish (Salmon, Grouper, Whitefish,Mackerel,..)				tbsp/pcs/slice
36.	Other seafoods (Prawns, Crabs,Shrimps, Squids,..)				tbsp/pcs/slice
37.	Processed meat (Sausages, Nuggets,Fish balls, Meat balls,..)				tbsp/pcs/slice
38.	Egg				tbsp/pcs/egg
39.	Others:				

No.	Food Items	Frequency (times)			Portion Size
Dairy Products		**Per month**	**Per Week**	**PerDay**	
40.	Breast milk				Cup(120 ml)/ml
41.	Formula milk powder				Cup(120 ml)/ml
42.	Milk (Fresh, Hi fat, Low fat, Pasteurised, UHT,..)				Cup(120 ml)/ml
43.	Fermented (Yogurt, Yakult, probiotics drinks, Yogurt drink,..)				Cup(120 ml)/ml
44.	Cheese				pcs/slice
45.	Others:				

No.	Food Items	Frequency (times)			Portion Size
Snacks		**Per month**	**Per Week**	**PerDay**	
46.	Bun				tbsp/pcs/bun
47.	Dim sum				tbsp/pcs/bun
48.	Cake (Pancakes, Muffins, Tart, Waffles, Fruit cake, Nuts cake,..)				tbsp/pcs/slice
49.	Cookies, Wafers, Biscuits				tbsp/pcs/slice
50.	Sweet (Candy, Jelly, Chocolate,Fritters, Pudding,..)				tbsp/pcs/slice
51.	Nuts (Peanuts, Almonds,..)				tbsp/pcs
52.	Traditional snacks (Klepon, Onde onde, Panada, Getuk, Surabi, Cireng,Bika ambon, Lemper, Dadar gulung,..)				tbsp/pcs/slice
53.	Preserved Fruits				tbsp/pcs/slice
54.	Others:				

No.	Food Items	Frequency (times)			Portion Size
Beverages		**Per month**	**Per Week**	**PerDay**	
55.	Soft drinks				Cup(120 ml)/ml
56.	Non-dairy milk drinks				Cup(120 ml)/ml
57.	Fruit juices				Cup(120 ml)/ml
58.	Milk shakes (Milo, Lassi,..)				Cup(120 ml)/ml
59.	Caffeinated (Coffee, Tea,...)				Cup(120 ml)/ml
60.	Water				Cup(120 ml)/ml
61.	Others:				

No.	Food Items	Frequency (times)			Portion Size
	Fast foods	**Per month**	**Per Week**	**PerDay**	
62.	Burger				tbsp/pcs/slice
63.	Fries (French fries, Hash brown,Wedges, Chips,...)				tbsp/pcs/slice
64.	Fried chicken				tbsp/pcs/slice
65.	Potato dished (Mashed potato,Whipped potato,..)				tbsp/tsp
66.	Others:				

No.	Food Items	Frequency (times)			Portion Size
	Seasoning	**Per month**	**Per Week**	**PerDay**	
67.	Sauce (Ketchup, Sambal, Applesauce,..)				tsp/tbsp/drop
68.	Spreads (Butter, Nutella, Honey,..)				tsp/tbsp/drop
69.	Savory (Soy sauce, Oyster, Cookingsauce,..)				tsp/tbsp/drop
70.	Others:				
	Supplements				
71.	Vitamins				tsp/tbsp/drop
72.	Others: (Probiotics supplements,DHA,..)				tsp/tbsp/drop

Appendix Text 2: Food Frequency Questionnaire for gut microbiota profile study (II)

Project ID no: _____

Age: _____

Race: Chinese / Malay / Indian / Eurasian /Others: _____

Gender: M / F

Height (in m): _____

Body weight (in kg): _____

Body Mass Index (BMI): _____

BMI = Mass(in kg)/ Height x Height (in m)

Food Item		Number of times eaten				
How often do you eat the following:		Never	Per Month	Per Week	Per Day	Average amount per serving
Bread						
1	White bread					------ slices:
2	Whole meal bread					------ slices:
3	Bread with fruits and nuts					------ slices:
Bread spreads used						
4	Butter					------ tsp:
5	Margarine					------ tsp:
6	Peanut butter					------ tsp:
7	Jams/honey					------ tsp:
8	Kaya					------ tsp:
Other types of breads						
9	Roti prata/ murtabak					----- slices:
10	Chapati/ Thosai / naan					----- slices:
11	Bread buns with coconut/ meat fillings					----- buns:
12	Bread buns with sweet filling (strawberry, kaya etc)					----- buns:
Cereal						
13	Plain/ flavoured breakfast cereal					----- dsp:
14	Oats/ oatmeal (raw)					----- dsp:
Rice and porridge						
15	Plain cooked white rice					----- dsp:
16	Plain porridge (cooked with white rice only					----- dsp:
17	Fried rice/ Claypot rice					----- dsp:
18	Chicken/duck rice					----- dsp:
19	Nasi Briyani/Nasi Lemak					----- dsp:
20	Flavoured porridge (eg., chicken, pork, duck, fish)					----- dsp:

Food Item		Number of times eaten				Average amount per serving
How often do you eat the following:		Never	Per Month	Per Week	Per Day	
Soup noodles						
21	Soup rice noodles (e.g. beehoon, kway teow, hor fun) with beef/ chicken/ fishball					----- dsp:
22	Soup wheat noodles (e.g.mee, udon, ramen) with beef/ chicken/ fishball					----- dsp:
Dry Noodles						
23	Dry rice noodles (e.g. beehonn, kway teow, hor fun) with beef/ chicken/ fishball					----- dsp:
24	Dry wheat noodles (e.g.mee, udon, ramen) with beef/ chicken/ fishball					----- dsp:
Fried Noodles						
25	Fried beehoon (rice noodle)					----- dsp:
26	Fried wheat noodle (e.g. Hokkien mee, mee goreng)					----- dsp:
Noodles in gravy						
27	Rice noodle in gravy (e.g. mee siam, hor fun, laksa)					----- dsp:
28	Wheat noodle in gravy (e.g. mee rebus)					----- dsp:
29	Instant noodles					----- dsp:
30	Boiled noodles/ spaghetti/ pasta (plain)					----- dsp:
31	Boiled noodles/ spaghetti/ pasta with cream white sauce					----- dsp:
Soups						
32	Cream soup					----- dsp
33	Clear soup/ broth					----- dsp

Food Item		Number of times eaten				
How often do you eat the following:		Never	Per Month	Per Week	Per Day	Average amount per serving
Spices/ Herbs						
34a	Chili Powder					NA
34b	Curry Powder					NA
34c	Ginger					NA
34d	Parsley					NA
34e	Tumeric					NA
34f	Coriander					NA
34g	Rosemary					NA
34h	Dill weeds					NA
34i	Mint Leaves					NA
34j	Others:					NA
Seasonings/ Sauces						
35a	Onion					NA
35b	Peppers					NA
35c	Chilli sauce					NA
35d	Others:					NA
Vegetables and Bean curd						
	Pale green leafy vegetable (cabbage, pak choy, lettuce, beansprouts, cauliflower etc.)					
36	Stir fried, plain					----- dsp:
37	Raw/ steam/ in soup					----- dsp:
	Dark green leafy vegetable (spinach, kai lan, chye sim, kangkong, broccoli etc.)					
38	Stir fried, plain					----- dsp:
39	Raw/ steam/ in soup					----- dsp:
	Tomatoes, carrots, re/yellow peppers					
40	Stir fried, plain					----- dsp:
41	Raw/ steam/ in soup					----- dsp:
	Legumes/ pulses eg., beans and peas					
42	Stir fried, plain					----- dsp:
43	Dried legumes (eg. Dhal, dried beans) in gravy					----- dsp:
44	Raw/ steam/ boiled					----- dsp:

Food item		Number of times eaten				
How often do you eat the following:		Never	Per Month	Per Week	Per Day	Average amount per serving
	Mixed vegetables					
45	Stir fried, plain					----- dsp:
46	Battered deep fried (e.g. tempura)					----- dsp:
47	Curry/ lemak					----- dsp:
48	Raw/ steamed/ in soup/ Rojak					----- dsp:
	Tofu and Bean curd					
49	Fried					----- dsp:
50	Steamed/ in soup					----- dsp:
	Roots/ Stems (potato, sweet potato, corn etc)					
51	Stir fried					----- dsp:
52	Soup with meat stews/stocks					----- dsp:
	Preserved Vegetables					
53	E.g. Chye sim, olives					----- dsp:
	Fermented Vegetables					
54	E.g. Kimchi, Tampeh, Natto					----- dsp:
Poultry without skin e.g. Chicken/Duck						
55	Stir fried/ stew/ braised/ roasted					----- dsp:
56	Pan deep fried					----- dsp:
57	Steamed					----- dsp:
Meat eg. Beef/ Pork/ Mutton						
	Meat-lean					
58	Stir fried/ stew/ braised/ roasted/ grilled/ BBQ					----- dsp:
59	Pan fried/ deep fried					----- dsp:
60	Steamed/ in soup					----- dsp:
	Meat preserved/ cured/ BBQ					
61	Sausages, please specify type (eg. Chicken, ham or pork)					-----
62	Ham/ Bacon/ Canned (eg. Luncheon meat, corned beef)					----- slices
63	Liver and other innards					----- dsp:
64	Meat jerky (eg. BBQ meat, Ba Kua, Beef stick)					----- dsp:

	Food item	Number of times eaten				
	How often do you eat the following:	Never	Per Month	Per Week	Per Day	Average amount per serving
Fish/ Seafood						
	Non-oily fish e.g.					
65	Stir fried/ grilled					----- dsp:
66	Pan fried/ deep fried/ deep fried with batter					----- dsp:
67	Steamed/ Assam pedas					----- dsp:
68	Coconut curry					----- dsp:
69	Canned (e.g. tuna)					----- dsp:
	Other seafood (e.g. prawns)					
70	Stir fried/ grilled					----- dsp:
71	Pan fried/ deep fried/ deep fried with Batter					----- dsp:
72	Steamed/ Assam pedas					----- dsp:
73	Coconut curry					----- dsp:
74	Raw seafood (e.g salmon, octopus)					----- dsp:
Whole eggs (including salted and century eggs)						
75	Boiled/ poached/ in soup/ steamed					----- dsp:
76	Fried/ scrambled					----- dsp:
	Egg whites, only					
77	Boiled/ poached/ in soup/ steamed					----- dsp:
78	Fried/ scrambled					----- dsp:
Salad Dressings						
79	Creamy dressing-regular (eg. Thousand island, mayonnaise, salad cream etc.)					----- dsp:
80	Creamy dressing-light/ low fat					----- dsp:
81	Oil-based dressing					----- dsp:

Food item		Number of times eaten				
How often do you eat the following:		Never	Per Month	Per Week	Per Day	Average amount per serving
Fruits						
82	Orange/red/yellow fresh fruits					---- pieces: or Proportion of fruits:
83	Other fresh fruits					----- Proportion of 1 whole fruit:
84	Fresh fruit juice					----- mls
85	Banana					----- pieces:
86	Canned fruits					----- dsp:
87	Mixed fruits (dried)					----- pieces:
Nuts						
88	Dry roasted					----- dsp:
89	Fried					------ dsp:
Milk and Dairy products						
90	Full cream milk (fresh, UHT, powder)					----- mls
91	Low fat milk (fresh, UHT, powder)					----- mls
92	Skimmed milk (fresh, UHT, powder)					----- mls
93	Low fat cheese					----- slices
94	Regular yoghurt					------ dsp:
95	Low fat yoghurt (including frozen yoghurt)					------ dsp:
96	Cheese					----- slices
97	Cheese spread					----- tsp or triangles
98	Others: (eg. Yakult, Vitagen, Lassi, Yoghurt drink, Fruit milk shake) Please specify ----------					----- mls
Soya Products						
99	Soya milk (fresh/ packet/ can)					----- mls
100	Soya Beancurd					----- dsp:

Food item		Number of times eaten				Average amount per serving
How often do you eat the following:		Never	Per Month	Per Week	Per Day	
Desserts/ Local snacks						
	Dessert in soup					
101	With coconut milk/ cream (eg. Bubor cha cha)					----- dsp:
102	Without coconut milk (eg. Cheng teng, green bean soup tau suan)					----- dsp:
	Kueh Kueh steamed					
103	With coconut/coconut milk/coconut cream (e.g. kuek sarlat, kueh dadar, putu mayam, idli)					----- pieces:
104	Without coconut milk (kuek tutu, soon kway)					----- pieces:
	Others					
105	Fried snacks (e.g. you tiao goring pisang, Indian rojak) Please Specify:					----- pieces:
106	Dim Sum- steamed (e.g. chee cheong fun, dumplings, rice dumplings)					----- pieces:
107	Dim sum- fried/deep fried (e.g. fried carrot cake, wanton, char siew					----- pieces:
108	Sweet Indian snacks (e.g. burfi, halwa)					----- pieces:
Biscuits, Pastries and Cakes						
109	Plain Biscuits					----- pieces:
110	Cream filled biscuits/shortbread					----- pieces:
111	Puff/flaky pastries (croissants, baked curry puffs etc)					----- pieces:
112	Plain butter cake/fruit cake					----- slices
113	Sponge cakes					----- slices
114	Cream cakes					----- slices

Food item		Number of times eaten				
How often do you eat the following:		Never	Per Month	Per Week	Per Day	Average amount per serving
Tibits/ Snacks						
115	Fried salty snacks (crisps, prawn crackers, keropok, salted biscuits etc)					----- pieces
116	Ice cream					----- dsp:
117	Chocolate					------ squares:
Sweetened Beverages						
118	Sweetened beverages (e.g. soft drinks, packet drinks, canned or bottled drinks)					----- mls
Other Beverages						
119	Coffee without sugar					----- mls
120	Coffee with sugar					----- mls
121	Tea without sugar					----- mls
122	Tea with sugar					----- mls
123	Green tea (unfermented tea)					----- mls
124	Black tea (fully unfermented tea)					----- mls
125	Oolong tea (partially fermented tea)					----- mls
126	Malt beverages (e.g. hot chocolate, Horlicks, Milo, Ovaltine)					----- mls
Alcoholic drinks						
127	Beer/Stout					----- mls
128	Wine					----- mls
129	Hard Liquor					----- mls
Vitamins and supplements						Quantity, duration, and brand

Appendix Text 3: Food Frequency Questionnaire (III)

Project ID no: _____

Age: _____

Race: Chinese / Malay / Indian / Eurasian /Others: _____

Gender: M / F

Height (in m): _____

Body weight (in kg): _____

Body Mass Index (BMI): _____

BMI = Mass(in kg)/ Height x Height (in m)

Section A (Personal information)		Yes	No	If yes, please specify
1.	Are you currently taking any medication within the past 7 days?			
2.	Has your diet changed over the past year? (EG: became a Vegetarian)			
3.	Do you have any dietary requirements? (EG: Vegetarian, Halal, Kosher, etc)			

Section B (Consumption of fermented food products)		Yes	No	If yes, how often?
1.	Do you consume fermented vegetables? (EG: Kimchi, pickles, etc)			_____time(s) per week
2.	Do you consume fermented dairy products? (EG: Yogurt, cheese, fermented milk, etc)			_____time(s) per week
3.	Do you consume fermented beverages? (EG: Wine, stout beer, sakae, yakult, etc)			_____bottle(s)/cup(s) per week
4.	Do you consume fermented condiments? (EG: Soya sauce, sour sauce, tabasco sauce, vinegar, ketchup)			_____time(s) per day
5.	Do you consume fermented snacks? (EG: Chocolate, probiotic ice cream, probiotic cheesecake, nata de coco etc)			_____packet(s) per week
6.	Do you consume other Types of fermented food that does not fit into the categories in questions 1 - 5? (EG: Cod Liver Oil (Traditional one), fermented tofu, Katuobushi (the topping on takoyaki))			_____time(s) per week
7.	Others, please specify:			

Section C (Consumption of carbohydrates, proteins and fats)		Please list them down in this column	
1.	Where is your main source of carbohydrate? (EG: Wheat-based bread, cereal, rice, pastries, noodles, etc)		____time(s) per week ____time(s) per week ____time(s) per week ____time(s) per week
2.	Where is your main source of protein? (EG: Chicken, duck, beef, pork, mutton, seafood, soy-based products, legumes, eggs, etc)		____time(s) per week ____time(s) per week ____time(s) per week ____time(s) per week
3.	Where is your main source of fat? (EG: Nuts, seeds, fish, plant-based oils, dairy products, etc)		____time(s) per week ____time(s) per week ____time(s) per week ____time(s) per week
4	What Type of oil do you usually consume at home? (EG: olive oil, canola oil, sunflower seed oil, etc)		____tsp(s) per day
5.	Do you consume supplements? (EG: Vitamin tablets, protein shake) If yes, please state the supplements which you are taking.		_____pill(s)/tablets/ cups per day

Section D (Consumption of vegetables, fruits, nuts)		Please list them down in this column	
1.	Vegetables		____time(s) per week ____time(s) per week ____time(s) per week ____time(s) per week
2.	Fruits		____time(s) per week ____time(s) per week ____time(s) per week ____time(s) per week
3.	Nuts		____time(s) per week ____time(s) per week ____time(s) per week ____time(s) per week

Section E (Consumption of fats)		Yes	No	If yes, how often?
1.	Do you eat deep fried food?			_____time(s) per week
2.	Do you eat pan fry food?			_____time(s) per week
3.	Do you eat steamed food?			_____time(s) per week
4.	Do you eat meat with skin?			_____time(s) per week
5.	Do you often opt for fatty parts of meat?			_____time(s) per week (Include examples)
6.	Others, please specify:			

Section F (Consumption of beverages)		Yes	No	If yes, how often?
1.	Do you drink tea?			_____cup(s) per day
2.	Do you drink coffee?			_____cup(s) per day
3.	Do you drink soft drinks? (EG: Ribena, ice lemon tea, coke)			_____bottle(s)/can(s) per week
4.	Do you drink alcohol? (EG: Vodka, whiskey, wine, champagne, beer)			_____glass(es) per week
5.	Do you drink fruit juices?			_____cup(s) per week
6.	Do you drink any dairy products? (EG: Milk)			_____cup(s) per week
7.	Others, please specify:			

Section G (Consumption of fast food)		Yes	No	If yes, how often?
1.	Do you consume fast food? (EG: French fries, burger, pizza)			_____time(s) per week

Section H (Consumption of spice, herbs and condiments)		Yes	No	If yes, how often?
1.	Do you consume spices? (EG: Chilli, nutmeg, cinnamon)			_____time(s) per day
2.	Do you consume herbs? (EG: Ginseng, basil, oregano)			_____time(s) per week
3.	Do you consume condiments? (EG: Ketchup, mustard, BBQ sauce, sour cream, thousand island, mayonnaise)			_____time(s) per day

Original Publications

Khine, W.W.T., Rahaya, E.S., See, T.Y., Kuah, S., Salminen, S., Nakayama, J., Lee, Y-K. (2020)
Indonesian children faecal microbiome from birth until weaning was different from microbiomes of their mothers.
Gut Microbes

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2020, VOL. 00, NO. 00, 1–19
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Indonesian children fecal microbiome from birth until weaning was different from microbiomes of their mothers

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ABSTRACT

Gastrointestinal (GI) microbiota play an important role in human health and wellbeing and the first wave of gut microbes arrives mostly through vertical transmission from mother to child. This study has undertaken to understand the microbiota profile of healthy Southeast Asian mother-infant pairs. Here, we examined the fecal, vaginal and breast milk microbiota of Indonesian mothers and the fecal microbiota of their children from less than 1 month to 48 months old. To determine the immune status of children and the effect of diet at different ages, we examined the level of cytokines, bile acids in the fecal water and weaning food frequency. The fecal microbiota of the children before weaning contained mainly *Bacteroides* and *Bifidobacterium*, which presented at low abundance in the samples of mothers. After weaning, the fecal microbiome of children was mainly of the *Prevotella* type, with decreasing levels of *Bifidobacterium*, thus becoming more like the fecal microbiome of the mother. The abundance of infant fecal commensals generally correlated inversely with potential pathogens before weaning. The fecal *Bifidobacterium* in children correlated inversely with the consumption of complex carbohydrates and fruits after weaning. The specific cytokines related to the proliferation and maturation of immunity were found to increase after weaning. A decreasing level of primary bile acids and an increase of secondary bile acids were observed after weaning. This study highlights the change in the GI microbiota of infants to adult-type microbiota after weaning and identifies diet as a major contributing factor.

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Introduction

The human body harbors an abundance of microbes on all mucosal surfaces. The composition and activity of these microbes form a symbiosis with the human host.[1–4] The mature human gastrointestinal (GI) microbiome type has been categorized into two operational types, determined largely by diet, geographic location, and lifestyle: the *Bacteroides-Bifidobacterium* type and *Prevotella* type.[5–9] The former type is predominant among Europeans, North Americans, and Eastern Asians,[5,9–11] whereas the latter is common among Southeast Asians, Mongolians, and Africans.[5,10,12] Mothers transfer their microbiomes to their children during pregnancy and delivery.[13–16] After birth, the vertical mother-to-infant transmission takes place mainly through the gut, the vagina, the breast milk, the oral cavity and the skin.[16–23] Vaginally delivered newborns of Western mothers have been reported to possess an identical GI microbiome type – the *Bacteroides-Bifidobacterium* type – to that of the mother,[17,22–25] although the relative abundance of the microbiota changes in the first year of life.[19,24,26–28] The GI microbiota, particularly *Bifidobacterium*, play an important role in health and disease programming for later stages of life and in modulating the development of the immune system, mental capacity and other physiological functions.[29–32] This implies that a hereditary composition of the microbiome of infants ensures the health and wellbeing of the children. Since Southeast Asians with the *Prevotella* type microbiome have low levels of *Bifidobacterium*,[8,10] this also implies potential health hazard for infants and thus warrants

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special attention. However, there is no Southeast Asian study on the relationship between the microbiomes of mothers and infants. Thus, the aim of this study is to track GI (as reflected in fecal) microbiota profiles among Indonesian mothers and their vaginally delivered, full-term, breastfed infants from birth (the first month of life) to 48 months old. This study addresses microbiome development among natural, full-term, breastfed infants, since their microbiome constitution is known to be influenced by route of birth,[33–35] antibiotics administration during delivery,[36,37] gestational age,[38,39] type of feeding,-[16,40,41] introduction of solid foods[8,11,12] and environmental factors.[42] Unexpectedly, we found that the newly born infant (before weaning) had a *Bacteroides-Bifidobacterium* microbiome type, which was different from their mothers' *Prevotella*-type and that the transition from *Bacteroides-Bifidobacterium* to *Prevotella* occurred during weaning.

Results

Fecal microbiota profiles of infants and their mothers

As shown in Figure 1a, the infants' fecal microbiota profiles were clustered separately from those of the mothers ($p < .05$) across all the age time points. The microbiota profiles of mothers were highly similar across all infant age groups (Figure 1b). On the other hand, there was significant variation in the fecal microbiota profiles of the children across the age groups (Figure 1b), including a reduction in the relative abundance of *Bifidobacterium, Bacteroides, Klebsiella*, and bacteria from the family *Enterobacteriaceae* along with increasing age. The mean abundance of *Bifidobacterium* was 2.5% ± SD 4.5% of total operational taxonomic units (OTUs) among the mothers in the study. From 6-<12 months onwards, the genera *Prevotella, Blautia, Faecalibacterium*, and *Ruminococcaceae*

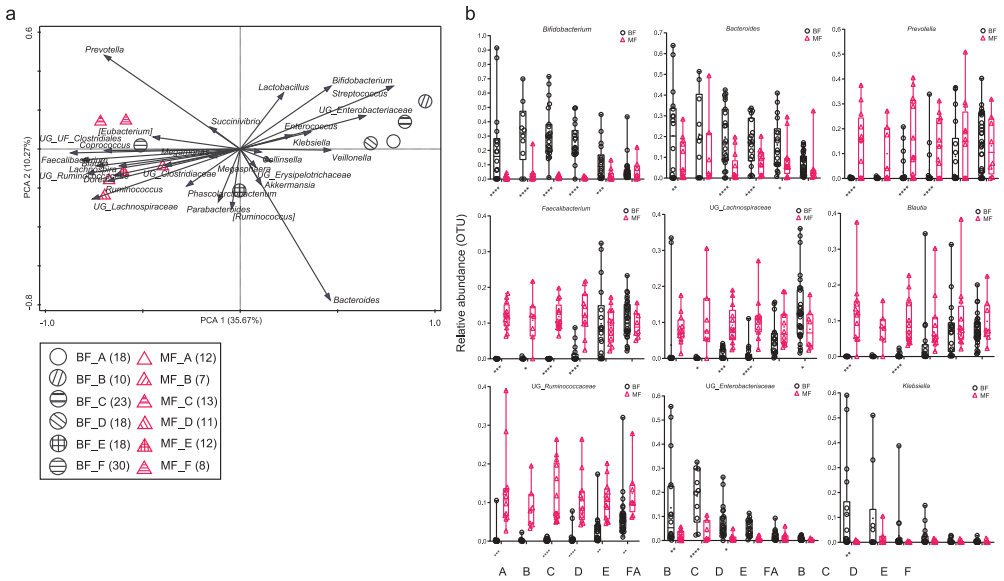

Figure 1. (a and b): Square root linear-contrast PCA of the fecal microbiome of children and mothers for children's ages of less than 1 month to 48 months. (a) The biplot shows the weight and direction of fecal bacterial genera and environment vectors in the Bray-Curtis distance matrix. Samples from each group are represented by different colors and symbols. Different patterns inside the symbols represent the different age groups. The numbers of mothers and children are provided in parentheses in the legend. (b) Comparison of relative abundance of nine bacterial genera among more than 1% of total OTUs in fecal samples of children and mothers across the age of less than 1 month to 48 months of children. The bacteria, which were significantly different between fecal samples of children and mothers, are represented as **** $p < .0001$, *** $p \geq 0.0001 - < 0.001$, ** $p \geq 0.001 - < 0.01$, * $p < .05$. BF = fecal samples of children, MF = fecal samples of mothers. A = less than 1 month old (BF = 18, MF = 12), B = 1–<3 months old (BF = 10,MF = 7), C = 3–<6 months old (BF = 23, MF = 13), D = 6–<12 months old (BF = 18, MF = 11), E = 12–<24 months old (BF = 18, MF = 12), F = 24–48 months old (BF = 30, MF = 8).

were detected in the fecal samples of the children. The abundance of these microbiota in the first 6 months of life was low (below 1% of total OTUs). After 6 months, the children started weaning. From this point, the children's fecal bacteria profiles appeared to shift toward their mothers' profile, with less microbiota showing differences in abundance between children and mothers. The profiles of the children and mothers were clustered at the period 24–48 months (Figure 1a). The most common OTUs at this period included *Prevotella, Lachospiraceae, Faecalibacterium, Blautia, Ruminococcaceae, Bacteroides, Bifidobacterium, Enterobacteriaceae* and *Klebsiella*.

The microbiota of mothers' and children's feces during the children's first month of life shared 5.2% (Table 1). The shared OTUs of the children's and mothers' fecal microbiota increased after weaning (6-<12 months) from 10.6% to 34.3% at 24–48 months.

Vaginal microbiota profile of mothers

At none of the infant age groups were the fecal microbiota profiles of children close to the maternal vaginal microbiota profiles (Figure 2a, b), even though all the infants were vaginally delivered. The shared microbiota of children's feces in the first

Table 1. Shared OTUs between the mothers' samples and the fecal samples of the children (%).

Category	Among the fecal samples of mothers	Among the vaginal swabs of mothers	Among the breast milk samples of mothers
<1 month old group	5.2	9.2	11
1- <3 months old group	5.8	12.3	9.3
3- <6 months old group	6.6	10.1	10.1
6- <12 months old group	10.6	15	11.1
12- <24 months old group	18.5	8.3	-
24- 48 months old group	34.3	19.1	-

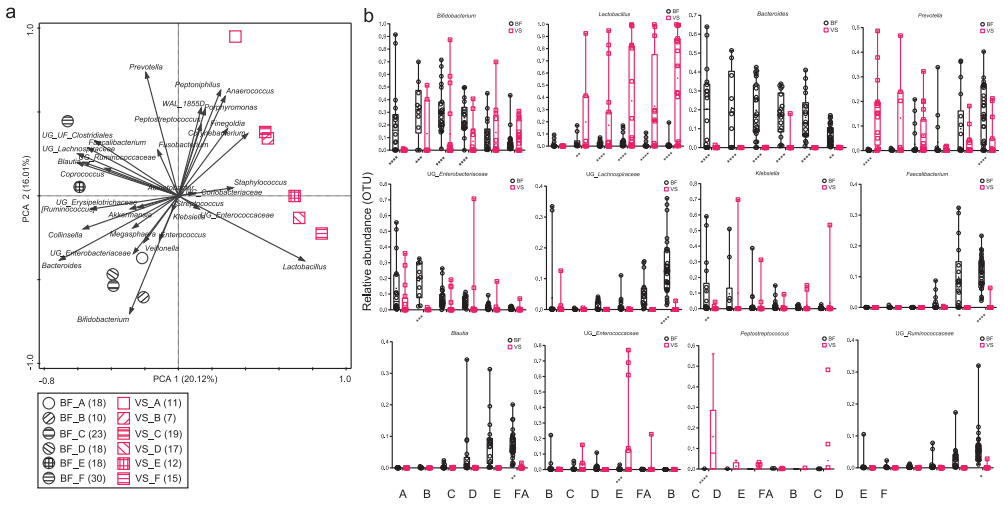

Figure 2. (a and b): Square root linear-contrast PCA of fecal samples of children and vaginal swab samples of mothers for children's ages of less than 1 month to 48 months. (a) The biplot shows the weight and direction of bacterial genera and environment vectors in the Bray-Curtis distance matrix. Samples from each group are represented by different colors and symbols. Different patterns inside the symbols represent the different age groups. The numbers of mothers and children are provided in parentheses in the legend. (b) Comparison of relative abundance of 12 bacterial genera among more than 1% of total OTUs in fecal samples of children and vaginal swab samples of mothers for children's ages of less than 1 month to 48 months. The bacteria, which were significantly different between fecal samples of children and vaginal swabs of mothers, are represented as **** $p < .0001$, *** $p \geq 0.0001 - < 0.001$, ** $p \geq 0.001 - < 0.01$, * $p < .05$. BF = fecal samples of children, VS = vaginal swab samples of mothers. A = less than 1 month old (BF = 18, VS = 16), B = 1–<3 months old (BF = 10,VS = 7), C = 3–<6 months old (BF = 23, VS = 19), D = 6–<12 months old (BF = 18, VS = 17), E = 12–<24 months old (BF = 18, VS = 12), F = 24–48 months old (BF = 30, VS = 15).

month of life and mothers' vagina was 9.2% (Table 1). The abundance of *Prevotella* in the vaginal microbiota was the highest when the children were less than 1 month old (Figure 2b) and gradually reduced thereafter. *Lactobacillus* was the most abundant throughout the different age groups apart from the first month of life. After 24 months of age, *Prevotella* made up about 10% of the total fecal OTUs of most of the children (66.7% of children).

Breast milk microbiota profiles of mothers

The microbiota profiles of mothers' milk were also clearly different from those of the children's feces at all ages (Figure 3a, b). The shared microbiota of children's feces during the first month of life and mothers' milk was 11.0% (Table 1). *Bifidobacterium* and *Bacteroides* (except 3-<6 months at 1%) each made up less than 1% of the total OTUs among the breast milk microbiota at all periods. *Staphylococcus* was the most abundant breast milk bacteria during the first month of life of infants, and subsequently, the abundance of *Streptococcus* was the highest.

Cytokines content of children's fecal water

Low levels of immune regulatory cytokines, namely IL-1β, −2, −5, −8, −12, TNF-α and IFN-ββ were found in infants' fecal water before weaning (<12 months), except IL-1β at 1–3 months. They were greatly enhanced after weaning up to 24 months (Figure 4). Among these cytokines, IL-12 levels peaked at 12-<24 months. The cytokine levels were generally reduced at 24–48 months. Anti-inflammatory cytokines, such as IL-4, −6 and −10, were low across all ages of children.

Fecal commensals and potentially pathogenic bacteria

The correlation between commensals and potentially pathogenic bacteria before weaning and after weaning is shown in Figure 5. The correlation index

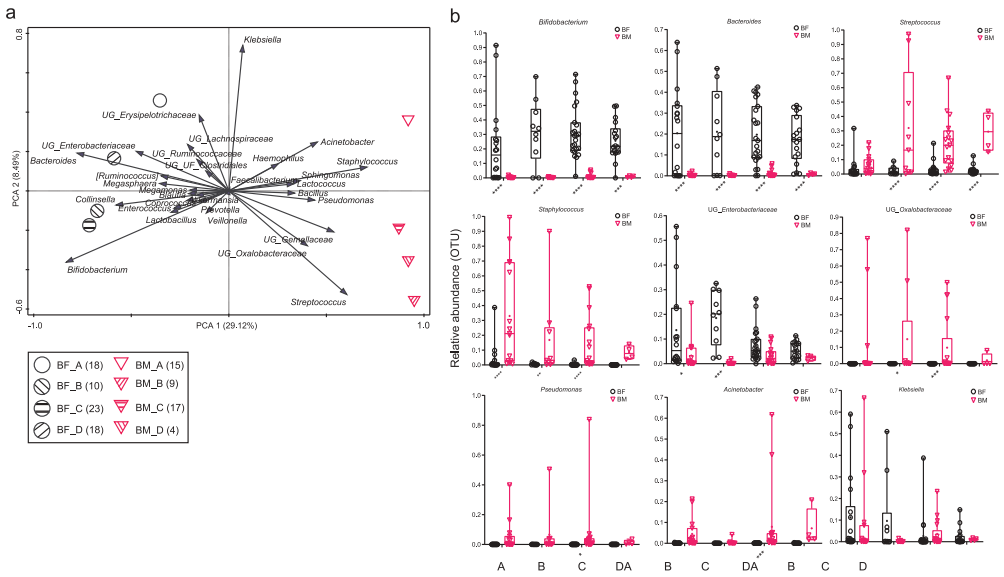

Figure 3. (a and b): Square root linear-contrast PCA of fecal samples of children and breast milk samples of mothers for children's ages of less than 1 month to 12 months. (a) The biplot shows the weight and direction of bacterial genera and environment vectors in the Bray-Curtis distance matrix. Samples from each group are represented by different colors and symbols. Different patterns inside the symbols represent the different age groups. The numbers of mothers and children are provided in parentheses in the legend. (b) Comparison of relative abundance of nine bacterial genera among more than 1% of total OTUs in fecal samples of children and breast milk samples of mothers for children's ages of less than 1 month to 12 months. The bacteria, which were significantly different between fecal samples of children and breast milk samples of mothers, are represented as **** $p < .0001$, *** $p \geq 0.0001 - < 0.001$, ** $p \geq 0.001 - < 0.01$, * $p < .05$. BF = fecal samples of children, BM = breast milk samples of mothers. A = less than 1 month old (BF = 18, BM = 15), B = 1–<3 months old (BF = 10, BM = 9), C = 3–<6 months old (BF = 23, BM = 17), D = 6–<12 months old (BF = 18, BM = 4).

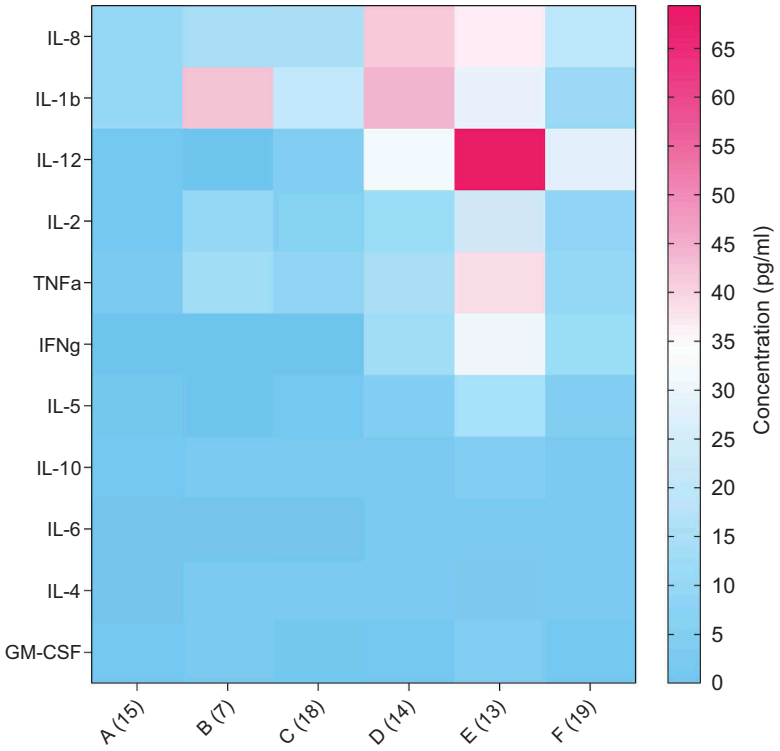

Figure 4. Comparison of concentration of cytokines in fecal water samples of children from the age of less than 1 month to 48 months. The data are expressed as the mean concentration in pg/ml. The number of samples is provided in parentheses at the X-axis. A = less than 1 month old, B = 1–<3 months old, C = 3–<6 months old, D = 6–<12 months old, E = 12–<24 months old and F = 24–48 months old.

indicates the degree of correlation between two groups of bacteria. A value of zero in a fecal commensal and potential pathogen pair implies no correlation in their abundance, whereas positive and negative values suggest positive and negative correlations. Interestingly, a higher percentage of commensal bacteria was found to be negatively associated with potential bacterial pathogens before weaning (Figure 5a, 46% negative correlation, with three pairs showing *p*-adjusted threshold of < 0.05) compared to after weaning (Figure 5b, 18% negative, with 12 pairs showing *p*-adjusted threshold of < 0.05). The correlation index was 4.85 before weaning and 30.97 after weaning.

Bile acids content in children's fecal water

High levels of free primary bile acids were detected in the fecal water of the infants before weaning (Figure 6a), but these levels progressively decreased to about a third of those levels after weaning. A reverse trend was observed for the level of secondary bile acids, which increased by 228 times after weaning (Figure 6b).

Correlation between the abundance of fecal microbiota and children's diet

The children consumed significantly more meats, vegetables, dairy products, and beverages as they aged (Figure 7). Several correlations were found between the abundance of specific fecal bacteria genera and the diet for the weaned infants (Table 2). *Bifidobacterium* was significantly associated with more than one food group. It was negatively associated with complex carbohydrates (mainly Indica rice) at 6-<12 months, with local fruits at 12-<24 months, and dairy products at 24–48 months.

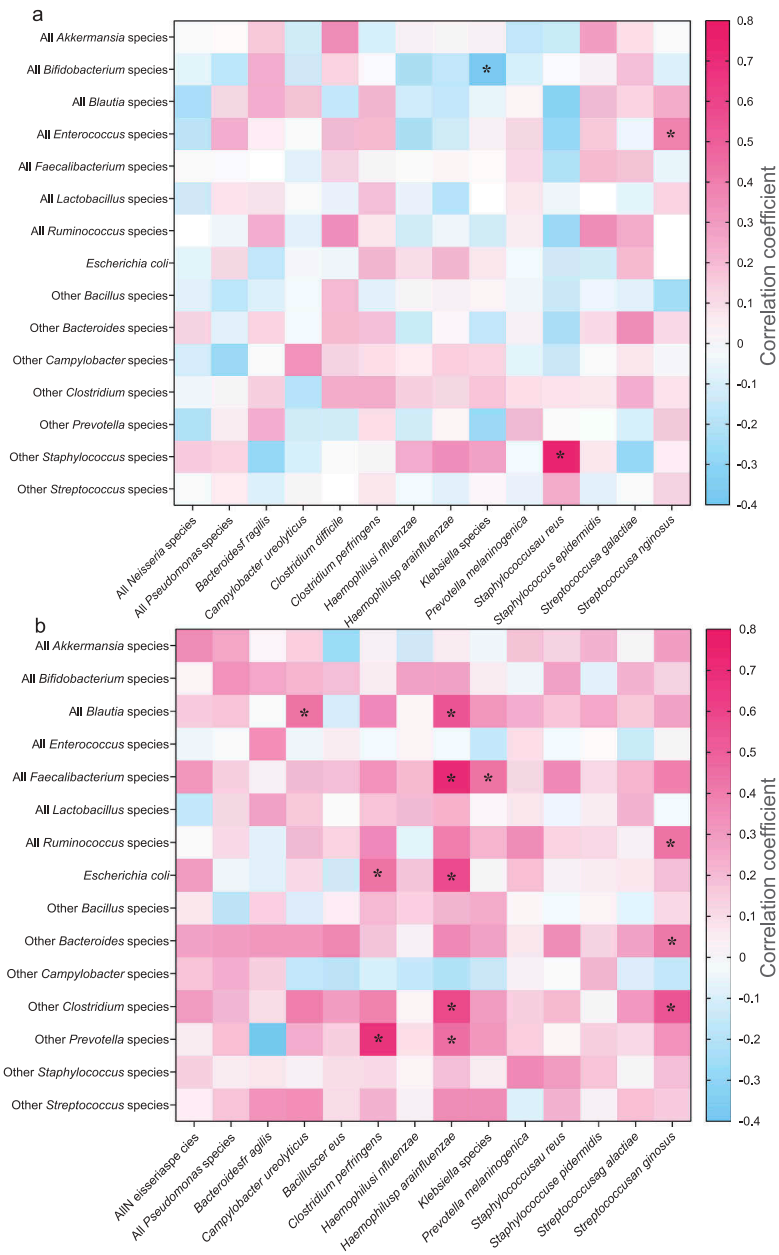

Figure 5. Correlation of known pathogens (X-axis) and known commensal gut species (Y-axis) at the species level of abundances (a) before and (b) after weaning (6–<12 months old) in the fecal samples of children. Spearman correlation coefficient values are plotted and presented as a heatmap. Positive and negative correlations are represented by different colors according to the gradient scales of the correlation coefficient. The significantly different correlations are represented as **** $p < .0001$, *** $p \geq 0.0001 - < 0.001$, ** $p \geq 0.001 - < 0.01$, * $p < .05$.

Bifidobacterium was positively associated with fast food and seasoning at 6-<12 months.

Besides their effect on *Bifidobacterium*, complex carbohydrates appeared to have the most influence on bacterial abundance at younger children (6-<24 months), including a positive correlation with *Lachnospiraceae, Ruminococcaceae*, Clostridiales, *Phascolarctobacterium* and *Lachnospira* among

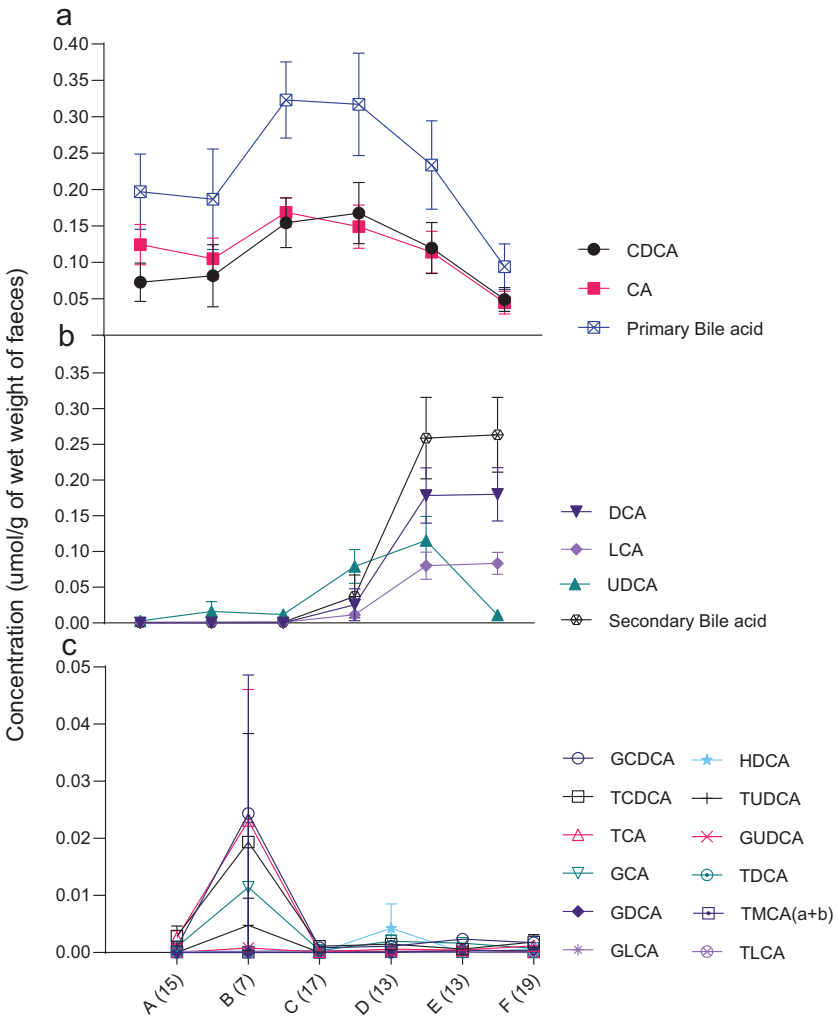

Figure 6. Comparison of concentration of free bile acids – (a) primary bile acids (CDCA, CA) (b) secondary bile acids (DCA, LCA, UDCA) (c) 12 bile acids – in fecal water samples of children from the age of less than 1 month to 48 months. The mean and standard error of the mean (SEM) are presented. Numbers of samples are described in parentheses at the X-axis. A = less than 1 month old, B = 1–<3 months old, C = 3–<6 months old, D = 6–<12 months old, E = 12–<24 months old, F = 24–48 months old age. CDCA = chenodeoxycholic acid, CA = colic acid, UDCA = ursodeoxycholic acid, DCA = deoxycholic acid, LCA = lithocholic acid, GCDCA = glycochenodeoxycholic acid, TCDCA = taurochenodeoxycholic acid, TCA = taurocholic acid, GCA = glycocholic acid, GDCA = glycodeoxycholic acid, GLCA = glycolithocholic acid, HDCA = hyodeoxycholic acid, TUDCA = tauroursodeoxycholic acid, GUDCA = glycoursodeoxycholic acid, TDCA = taurodeoxycholic acid, TMCA (a + b) = alpha- and beta-tauromuricholic acid, TLCA = taurolithocholic acid.

those aged 6-<12 months, and *Bacteroides, Ruminococcus, Akkermansia* and *Succinivibrio* among the 12-<24 months. A negative correlation between complex carbohydrates and *Phascolarctobacterium* was observed at 12-<24 months.

Apart from their effect on *Bifidobacterium*, local fruits appeared to have more influence on bacterial abundance at the older age (12-<48 months). A positive correlation was observed between fruit consumption and *Coprococcus* and *Ruminococcus* at 12-<24 months, whereas fruit consumption was negatively correlated with *Phascolarctobacterium* at 6-<12 months, *Lactobacillus* at 12-<24 months, *Ruminococcaceae, Akkermansia* and *Parabacteroides* at 24-<48 months. Other food groups also influenced

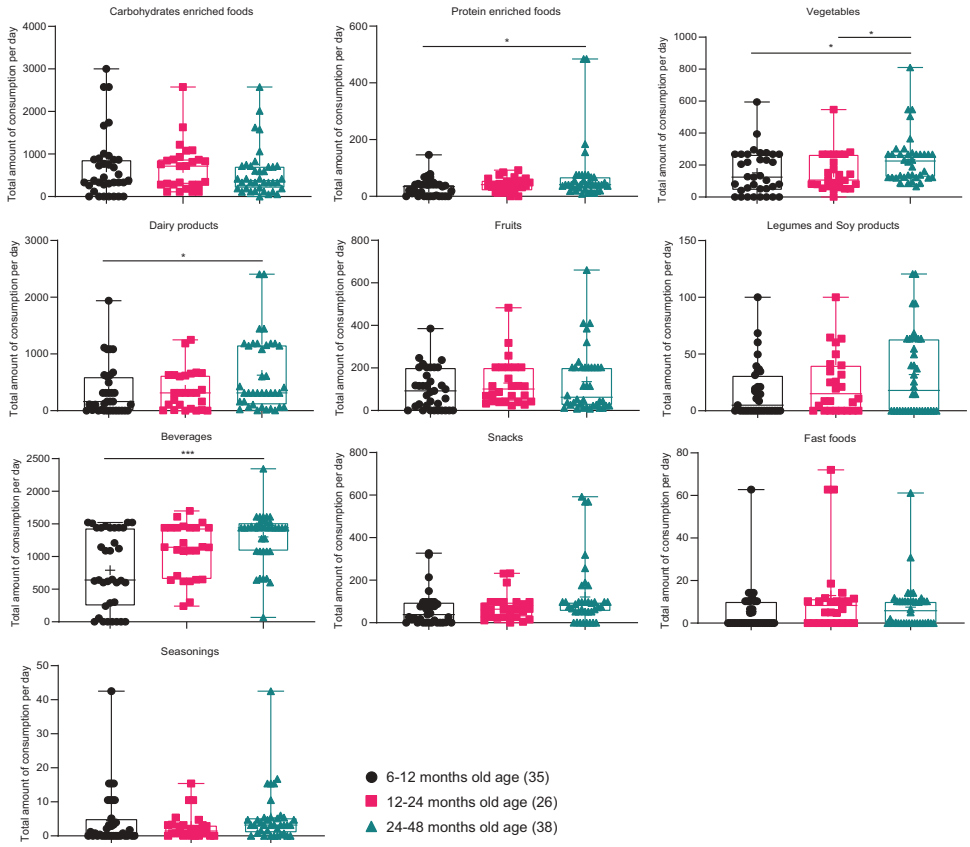

Figure 7. Comparison of the total amount of dietary consumption (g) per day of children aged 6–48 months. The foods, which were significantly different between three age groups of children, are represented as **** $p < .0001$, *** $p \geq 0.0001 - < 0.001$, ** $p \geq 0.001 - < 0.01$, * $p < .05$.

the fecal microbiota of children and are presented in Table 2.

Discussion

Bacteroides, Bifidobacterium, and *Enterobacteriaceae* were the dominant fecal bacteria in children during early life in this study, which has been reported in studies conducted in the Western countries.[19,24–26] The variation with age observed in the microbiota profile of the children's fecal samples was due to the appearance of certain genera in the older age groups. In the 6–12 months and 12–24 months age groups, *Prevotella, Blautia, Faecalibacterium, Lachnospiraceae*, and *Ruminococcaceae* were the genera found. At the same time, the relative abundance of *Bacteroides, Bifidobacterium, Enterobacteriaceae*, and *Klebsiella* diminished after weaning (6-<12 months) and stabilized at lower levels at 24–48 months.

The rapid (less than one month after birth) establishment of the children' GI microbiota as reflected in the fecal microbiota, from the mothers' minority GI microbiota (in this case, *Bacteroides* and *Bifidobacterium*) may indicate swift growth and expansion of the microbiota in the GI tracts of the newborns when the GI environmental conditions are favorable for their proliferation and colonization. This calls into question the reason for the delay in the establishment of *Bifidobacterium* and *Bacteroides* in Cesarean-delivered infants, even those who are breastfed.[24,43] It has been assumed that such delayed establishment is due to low vertical transmission from mother to

Table 2. Correlation between fecal bacterial genus abundance (> 1% of total OTUs) and amount of certain food groups children consumed per day at ages (a) 6–12 months, (b) 12–24 months and (c) 24–48 months.

(a) 6-12 months old

Bacteria	Complex Carbohydrates (g)	Vegetables (g)	Legumes and Soys (g)	Fruits (g)	Meats (g)	Dairy Products (g)	Snacks (g)	Beverages (g)	Fast Foods (g)	Seasoning (g)
Bifidobacterium	*-0.661	-0.241	-0.465	0.159	-0.196	0.023	0.159	0.597	**0.765	**0.807
Bacteroides	0.333	-0.059	0.172	-0.041	*0.733	-0.077	-0.296	-0.159	**-0.770	-0.462
Prevotella	-0.137	0.173	0.451	0.255	-0.419	-0.027	0.118	-0.018	0.234	0.098
Faecalibacterium	0.292	0.392	0.372	0.200	0.219	-0.301	-0.346	-0.364	-0.313	-0.364
UG_Lachnospiraceae	*0.692	-0.128	0.065	-0.492	0.009	-0.456	0.082	0.027	-0.214	-0.117
Blautia	0.606	0.542	-0.154	-0.223	0.187	-0.296	*0.697	-0.569	-0.124	-0.205
UG_Ruminococcaceae	*0.706	0.187	0.070	-0.551	-0.378	-0.323	0.023	-0.123	-0.084	-0.121
UG_Enterobacteriaceae	-0.583	-0.091	-0.023	0.583	-0.046	-0.182	-0.528	0.583	0.169	0.494
Klebsiella	-0.036	0.164	0.563	0.237	-0.328	0.428	-0.319	-0.282	-0.452	-0.536
[Ruminococcus]	0.456	-0.118	-0.484	-0.528	0.200	-0.137	0.064	-0.009	-0.258	0.014
UG_UF_Clostridiales	*0.715	0.542	-0.167	-0.314	0.187	*-0.706	0.351	-0.205	-0.065	0.140
Collinsella	-0.241	-0.478	-0.233	0.096	0.059	0.032	-0.123	0.251	0.114	0.051
Coprococcus	0.456	0.155	-0.242	-0.592	0.018	0.191	0.164	-0.446	-0.199	-0.191
Lactobacillus	-0.515	-0.333	0.279	0.333	0.050	0.551	-0.196	-0.077	-0.060	-0.238
Streptococcus	0.059	-0.278	-0.419	-0.506	-0.451	0.132	0.059	0.278	0.303	0.256
Ruminococcus	0.264	-0.146	0.321	-0.237	0.128	-0.191	-0.364	0.082	-0.164	-0.084
Megasphaera	0.146	0.501	*0.628	0.510	0.337	-0.219	-0.182	-0.273	*-0.636	-0.289
Akkermansia	-0.272	0.454	-0.083	0.291	-0.263	0.043	-0.244	0.110	-0.005	0.396
[Eubacterium]	0.100	0.146	-0.098	0.128	0.137	*-0.674	0.100	0.273	0.268	0.443
UG_Erysipelotrichaceae	0.460	0.342	0.070	-0.169	-0.014	-0.132	**0.788	-0.569	0.094	-0.354
Veillonella	-0.273	-0.528	0.233	0.128	-0.191	-0.027	-0.538	0.465	0.219	-0.042
Succinivibrio	-0.347	-0.100	0.445	0.379	-0.571	0.233	-0.210	0.233	-0.027	0.035
Enterococcus	0.137	0.128	-0.079	-0.237	0.091	-0.210	0.091	0.173	0.119	0.308
Dorea	0.454	0.107	-0.124	-0.285	-0.094	-0.340	0.130	0.094	-0.219	0.150
Megamonas	0.132	0.097	-0.454	-0.221	0.201	-0.430	0.325	0.241	0.263	0.598
Phascolarctobacterium	*0.696	-0.104	-0.421	** -0.78	0.293	-0.238	-0.214	0.030	-0.428	-0.036
UG_Clostridiaceae	0.000	-0.273	0.284	0.082	0.091	*-0.073	-0.538	0.146	-0.253	-0.131
Lachnospira	*0.633	0.396	0.181	0.050	0.351	-0.743	0.087	-0.169	-0.378	-0.163
Parabacteroides	0.208	0.153	-0.175	-0.263	0.103	-0.048	0.144	-0.071	-0.139	0.215

(b)12-24 months old

Bacteria	Complex Carbohydrates (g)	Vegetables (g)	Legumes and Soys (g)	Fruits (g)	Meats (g)	Dairy Products (g)	Snacks (g)	Beverages (g)	Fast Foods (g)	Seasoning (g)
Bifidobacterium	0.322	0.007	0.491	** 0.748	0.497	-0.273	-0.070	-0.140	0.068	-0.296
Bacteroides	*0.629	-0.252	0.516	-0.559	-0.147	-0.280	-0.476	-0.503	-0.196	-0.049
Prevotella	-0.119	-0.042	-0.174	0.000	0.231	0.014	0.322	0.007	-0.231	-0.056
Faecalibacterium	-0.133	-0.259	-0.196	0.524	0.021	0.063	0.413	0.084	0.046	0.134
UG_Lachnospiraceae	-0.168	-0.133	-0.082	0.357	-0.112	-0.070	-0.140	0.056	-0.399	-0.197
Blautia	-0.231	-0.308	-0.125	0.378	-0.133	0.277	0.280	-0.021	-0.288	0.000
UG_Ruminococcaceae	-0.280	-0.343	-0.256	0.469	-0.063	0.116	0.301	0.133	-0.342	-0.070
UG_Enterobacteriaceae	-0.042	-0.007	-0.018	-0.287	-0.266	-0.123	-0.385	0.329	0.000	0.148
Klebsiella	-0.301	0.168	-0.445	0.476	-0.524	0.067	-0.182	0.364	0.139	0.472
[Ruminococcus]	0.503	-0.245	0.473	-0.343	-0.406	-0.336	-0.524	-0.364	-0.484	0.014
UG_UF_Clostridiales	-0.357	-0.077	-0.274	0.329	-0.133	-0.077	-0.126	0.301	-0.484	-0.021
Collinsella	0.049	0.105	0.267	0.133	-0.028	-0.116	0.119	-0.077	0.075	0.359
Coprococcus	-0.287	-0.203	-0.481	*0.636	-0.091	0.126	-0.049	0.315	0.110	-0.070
Lactobacillus	0.413	-0.266	0.445	**-0.763	0.399	-0.399	-0.245	-0.245	-0.274	-0.387
Streptococcus	-0.021	0.196	0.238	-0.280	0.007	-0.578	*-0.671	0.476	0.224	0.021
Ruminococcus	*-0.699	0.084	*-0.655	**0.762	0.154	0.434	0.455	0.406	0.242	0.211
Megasphaera	0.147	-0.070	0.384	-0.469	0.042	-0.438	-0.182	0.196	-0.117	-0.387
Akkermansia	*0.696	0.007	*0.594	-0.580	0.174	-0.192	-0.058	*-0.711	0.048	-0.146
[Eubacterium]	-0.056	-0.105	-0.021	0.000	-0.175	0.312	-0.007	-0.119	0.199	0.570
UG_Erysipelotrichaceae	0.147	0.280	0.352	-0.315	0.392	-0.308	-0.154	-0.154	-0.146	-0.232
Veillonella	0.084	-0.014	-0.058	-0.441	0.112	0.109	-0.245	-0.063	0.285	0.000
Succinivibrio	*0.671	0.031	*0.730	-0.468	-0.491	*-0.594	-0.172	-0.242	-0.119	0.031
Enterococcus	-0.130	0.140	0.016	-0.291	0.501	0.154	0.039	0.105	0.164	-0.296
Dorea	-0.252	0.042	0.039	0.315	-0.091	0.196	0.294	0.091	-0.128	0.239
Megamonas	-0.396	0.411	-0.298	0.224	0.317	0.254	0.515	0.105	0.150	0.169
Phascolarctobacterium	*-0.637	**0.787	-0.484	0.057	0.374	0.488	0.146	0.224	0.391	0.523
UG_Clostridiaceae	-0.119	0.021	-0.231	0.175	-0.545	-0.161	*-0.587	0.385	0.182	0.423
Lachnospira	0.210	0.221	0.248	-0.032	0.116	-0.319	0.004	-0.168	-0.059	-0.226
Parabacteroides	0.242	-0.056	0.278	-0.133	-0.095	0.212	0.322	-0.424	-0.451	-0.081

(c) 24-48 months old

Bacteria	Complex Carbohydrates (g)	Vegetables (g)	Legumes and Soys (g)	Fruits (g)	Meats (g)	Dairy Products (g)	Snacks (g)	Beverages (g)	Fast Foods (g)	Seasoning (g)
Bifidobacterium	0.204	0.587	-0.025	-0.539	0.299	**-0.874	-0.443	0.395	0.610	0.683
Bacteroides	0.192	-0.048	-0.114	-0.096	0.168	0.072	-0.120	-0.048	0.122	-0.366
Prevotella	0.515	0.228	-0.660	0.299	0.299	0.299	0.228	-0.323	-0.220	-0.024
Faecalibacterium	0.156	-0.084	-0.736	0.084	0.132	0.467	-0.084	-0.012	-0.512	-0.366
UG_Lachnospiraceae	-0.431	-0.335	0.190	0.671	-0.048	*0.838	0.575	-0.431	-0.171	-0.244
Blautia	-0.599	0.168	0.507	0.311	0.000	0.120	0.287	-0.096	0.439	0.561
UG_Ruminococcaceae	0.503	0.287	-0.596	*-0.838	0.000	-0.695	**-0.862	0.575	-0.122	-0.049
UG_Enterobacteriaceae	0.036	0.180	0.444	-0.036	-0.275	-0.204	-0.012	-0.132	0.342	0.122
Klebsiella	-0.659	-0.419	0.330	-0.012	0.467	0.132	0.060	0.252	0.317	-0.122
[Ruminococcus]	0.144	-0.120	0.063	-0.048	-0.647	0.096	-0.120	-0.096	-0.415	-0.342
UG_UF_Clostridiales	-0.659	-0.659	0.317	-0.491	-0.156	-0.180	-0.419	0.659	-0.244	-0.366
Collinsella	0.395	0.563	0.038	-0.443	0.252	**-0.874	-0.299	0.228	0.586	0.610
Coprococcus	0.623	0.527	-0.114	-0.048	-0.359	-0.311	-0.072	-0.240	0.024	0.268
Lactobacillus	-0.024	-0.144	0.266	-0.168	*-0.838	-0.072	-0.216	0.048	-0.415	-0.220
Streptococcus	-0.395	0.084	0.596	0.204	0.299	-0.012	0.275	-0.132	0.732	0.317
Ruminococcus	0.228	-0.204	-0.355	-0.707	0.275	-0.515	-0.611	0.587	-0.122	-0.342
Megasphaera	0.554	0.675	-0.217	-0.434	0.410	*-0.747	-0.361	0.157	0.638	0.491
Akkermansia	-0.012	0.307	0.091	*-0.773	-0.135	**-0.920	-0.700	0.650	0.250	0.450
[Eubacterium]	0.084	0.204	-0.203	0.347	0.084	0.228	0.299	-0.252	-0.073	0.268
UG_Erysipelotrichaceae	0.120	0.144	-0.457	0.263	0.216	0.431	0.120	-0.216	-0.098	-0.073
Veillonella	0.719	0.479	0.076	0.335	-0.168	-0.048	0.359	-0.647	0.244	0.171
Succinivibrio	0.061	-0.503	0.195	0.135	*-0.773	0.282	0.135	-0.184	*-0.75	-0.575
Enterococcus	0.216	0.144	0.228	-0.359	-0.096	-0.575	-0.240	0.168	0.244	0.146
Dorea	0.299	0.228	0.165	0.132	-0.395	0.084	0.060	-0.371	0.098	-0.098
Megamonas	0.024	0.216	-0.596	-0.216	**0.886	-0.048	-0.263	0.287	0.366	0.098
Phascolarctobacterium	0.491	-0.036	-0.051	0.275	0.012	0.180	0.323	-0.443	-0.073	-0.293
UG_Clostridiaceae	0.108	-0.563	-0.216	-0.443	-0.156	-0.036	-0.419	0.347	-0.537	*-0.805
Lachnospira	0.024	-0.240	-0.101	0.120	0.599	0.144	0.216	-0.048	0.146	-0.220
Parabacteroides	0.036	-0.060	-0.355	**-0.946	0.252	*-0.755	**-0.874	**0.874	-0.024	-0.073

Spearman correlation coefficient (r) values are tabulated. The significant positive and negative correlations are shaded in pink and blue colors respectively. The significance level is indicated as follows: **** $p < 0.0001$, *** p ≥ 0.0001 – < 0.001, ** p ≥ 0.001 – < 0.01, * p ≥ 0.01- < 0.05.

child.[24,43] However, as this study shows, it does not require high inoculation for a bacterium to rapidly establish in the GI tract during early infancy. It is possible that Cesarean section medical procedure, itself could instead alter the GI environment, hindering the establishment of commensal bacteria.

Human milk oligosaccharides promote the growth and colonization of *Bifidobacterium* before weaning.[44–46] Furthermore, breast milk is high in fat,[47] which may induce secretion of bile acids in the GI, inhibit *Prevotella* and facilitate the proliferation of *Bacteroides* and *Bifidobacterium*, as reported in the literature.[48,49] High levels of free primary bile acids were detected in the fecal water of children before weaning (Figure 6). The changes in the GI microbiome after weaning resulted in the conversion of primary bile acids to secondary bile acids (Figure 6), which is supported by a study of Japanese infants.[50]

The function of *Bacteroides* and *Bifidobacterium* before weaning and the reason that they are prevalent in children' GI requires attention. In the case of Indonesians, *Bacteroides* and *Bifidobacterium* are less likely contribute to the digestion of nutrients in adulthood, as shown in the shift of major microbiota from *Bacteroides* and *Bifidobacterium* to *Prevotella* upon weaning (up to 48 months old) and low prevalence of *Bacteroides* and *Bifidobacterium* in mothers (adults).

In this study, cytokines, which are responsible for proliferation and maturation of immune cells and the immune system (IL-1β, −2, −5, −8, −12, TNF-α and IFN-β), were found to be upregulated after weaning (Figure 4). This implies that the active immunity of children develops around the weaning period. In a study of mice, *Bacteroides fragilis* colonization was found to suppress pro-inflammatory TH17 cell responses.[51] The abundance of *Bacteroides* found before weaning in this study suggests a possible down-regulation of pro-inflammatory cells.

Prevotella was the most abundant in the vaginal samples of mothers whose children were <1 month old (Figure 3b). The mothers may have inoculated their children heavily with *Prevotella*, but the bacterium may have been maintained in the children at a low level during the breastfeeding period and turned dominant after the children consuming the foods provided by the mothers upon weaning. Interestingly, the vaginal microbiota of European and North American mothers, whose GI microbiota are of the *Bacteroides-Bifidobacterium* type, are high in *Prevotella*,[16,19] although *Prevotella* does not have a role in high fat/protein European and North American diets after weaning.[8,11] The intrinsic factors for the establishment of *Prevotella* in the vaginas of mothers could be entrenched despite changes in dietary patterns in the developed country in recent years. The early human diet was largely vegetable-based, like the diet of Indonesians today.

Maternal microbiota transmission was still identified as the main source of children's GI microbiota as all the microbiota of the children could be found in mothers (Fig. 1–3). However, the high number of OTUs in mothers that were not shared with the children (Table 1) indicates that only a small proportion of the GI, vagina, and breast milk microbiota from the mothers were able to establish in the GI of their children at the early age. The predominant microbiota of the mothers did not have an advantage in colonizing the GI tracts of the children. One explanation for the colonization of the infants' GI by the mothers' nondominant strains may be the selective preference of specific functional genes of the infants.[23,52]

The GI microbiota may have been transmitted not only vertically but also horizontally.[53] Nevertheless, as this and other studies have found, the percentages of shared fecal OTUs between mothers and children increased with age (Table 1).[22,24] The fact that fecal samples of older children shared more OTUs with the vaginal swab of their mothers compared to those of younger children could reflect personal hygiene practices among the Indonesian subjects (Table 1).

The correlation index for fecal commensals and pathogens was 4.9 before weaning and 31.0 after weaning. This implies that commensal bacteria, in conjunction with the passive immunity provided by immunoglobulins and antibodies in the mothers' breast milk, may have suppressed and prevented overgrowth of potential pathogens. These commensals protect children from potential pathogens before the development of immunity around the time of weaning. This was particularly important for the survival of pre-weaned children in the early period of human history when hygiene was not a common practice and remains so in developing

countries. If this is the case, human pathogens of concern in the early history could be different from the potential pathogens detected in fecal samples in this study as *Bifidobacterium, Bacteroides* and *Prevotella* showed a negative correlation with the same groups of potential pathogens before weaning. *Vibrio cholera* is the most widespread fatal GI pathogen reported in developing countries but it was not identified in this study. Clostridiales have been reported to protect against GI colonization by bacterial pathogens in an animal model.[54] However, Clostridiales accounted for less than 1% of total OTUs in the first 6 months of life.

Bifidobacterium was negatively associated with complex carbohydrates, local fruits, and dairy products at various age groups. This study (Table 2) supports a previous finding[10] of a negative association of *Bifidobacterium* with the presence of resistant starch. The main staple carbohydrate of Indonesia is Indica rice, which is high in resistant starch. Its presence due to consumption of rice after weaning and persistence in the GI tract lead to the removal of free bile acids from the lumen, which may have enabled *Prevotella* to proliferate and reduced the abundance of *Bifidobacterium*, as proposed in the literature.[10]

Besides, *Prevotella* is a carbohydrate fermenter,[10,11] which would proliferate in the high carbohydrate diet of the Indonesians. Furthermore, local fruits and dairy products appear to have inhibited the proliferation of *Bifidobacterium* in a strong dose-dependent matter (Table 2). Local fruits may produce anti-microbial biomolecules for self-preservation in a high temperature environment which favored microbial proliferation.[55–57] In this case, *Bifidobacterium* could be one of the sensitive microbes, which needs to be further verified. This may explain the drastic decrease in the abundance of *Bifidobacterium* in the fecal samples of children after weaning and its low abundance in mothers (Figure 1). In special cases where children consumed more fast foods, the digestible carbohydrate in the wheat-based foods, such as buns, and in potato chips may lead to the positive correlation between fast foods and *Bifidobacterium* (Table 2).[10,58]

The health-promoting functions of *Bifidobacterium* observed in European, North American and East Asian children[29-32,43,54] may, in Indonesians, have been replaced by other commensal bacteria, such as *Enterococcus faecalis*.[59,60] At 24–48 months, a matured, balanced microbiome resembling that of the mothers may have developed in the children, because of the convergence of their diets. The immune system may have approached maturation at 24–48 months, leading to the downregulation of regulatory cytokines, namely IL-1β, −2, −5, −8, −12, TNF-α and IFN-γ (Figure 4).

Conclusions

The study suggests that:

(1) The establishment of the predominant bacteria in infants is relatively rapid (within one month after birth) and that it can be initiated by only a small number of seeding bacterial cells from the mothers, such as *Bacteroides* and *Bifidobacterium* which were parts of minority microbiota in all mother's samples (feces, breast milk, and vagina).
(2) The predominant microbiota of children before weaning are associated with intrinsic and extrinsic factors, such as bile concentration, and cytokines, and possibly milk oligosaccharides and mucin glycan (proposed in literature, not measured in this study) which may favor *Bacteroides* and *Bifidobacterium*.
(3) *Bacteroides* and *Bifidobacterium* are negatively associated with potential pathogens before weaning. Their roles in the protection against infectious diseases need to be verified in clinical studies.
(4) Dietary shift after weaning modifies the fecal microbiota of the children from *Bacteroides* and *Bifidobacterium* to *Prevotella*.
(5) Taken together, the data suggest that the children's microbiota profiles were largely determined by the GI environment and dietary components rather than maternal transfer. In other words, certain intrinsic and extrinsic factors may determine the preferred microbiota that colonize the GI tract in children. This has important implications for approaches to remediating GI microbiota dysbiosis.

Methods

Study design

Recruitment of subjects

Three hundred healthy Indonesian mothers and their children under 4 years old were recruited from three community health centers in Yogyakarta, Indonesia. The inclusion criteria for recruitment of subjects were that the children had been born by normal vaginal delivery, had no history of hospitalization for serious illness at birth and had been exclusively breastfed before weaning period (6-<12 months old age). Only mothers and children who had not received intrapartum antibiotics were recruited. A total of 157 mother-child pairs participated in the study after screening for the inclusion criteria. The mother-child pairs were categorized into six groups according to the age of the child: <1 month (25 pairs), 1-<3 months (10 pairs), 3-<6 months (23 pairs), 6-<12 months (35 pairs), 12–<24 months (26 pairs) and 24-<48 months (38 pairs).

Collection of samples and dietary questionnaires

Fecal and breast milk samples and lower vaginal swabs were collected from mothers and fecal samples were collected from their children. A one-time sample collection was conducted for each mother and child. Most of the mothers in the age group 12-<24 months had stopped lactating.

On the same day of samples were collected, subjects whose children were weaned (the 6-<12, 12-<24 and 24-<48 months groups) were given the weaning food frequency questionnaire (FFQ) (Text S1). In the questionnaire, the mothers were asked to record the frequency of consumption per day/week/month and the serving size of each food item taken by their children.

Collection of fecal samples from mothers and children

The subjects were asked to collect approximately 10 g (3–5 scoops using the provided spatula) of feces which was then suspended in a collection tube containing 2 ml of RNAlater® (Ambion Inc., USA).

Collection of vaginal swabs from mothers

Health-care personnel swabbed the lower part of the vaginal region of each subject with a sterile cotton swab which was then preserved in a tube containing 1 ml of Amies Transport solution.

Collection of breast milk samples from mothers

The nipples and areola of the mother's breast were cleaned with an alcohol swab, and approximately 5–10 ml of breast milk was collected in a sterile 50 ml tube.

Microbiome analysis

Sample processing and DNA extraction from samples

Fecal samples

Each fecal sample was further diluted 10 times by RNAlater® to make a fecal homogenate. 200 µl of each fecal homogenate was washed twice with 1x phosphate buffered saline (PBS). After washing, each fecal pellet was suspended with 300 ml of Tris-SDS solution. The mixture was transferred to a tube containing 0.3 g of glass beads (0.1 mm diameter), to which 500 ml of TE-saturated phenol (Sigma-Aldrich, USA) was added. A benchmark BeadBlaster 24 (Benchmark Scientific, Edison, USA) was used to mechanically break down the cells and the resulting lysate was centrifuged (15,000 rpm at 4°C for 5 minutes). The supernatant was transferred to a new tube containing 400 µl of phenol/chloroform/isoamyl alcohol (25:24:1) (Sigma-Aldrich, USA) was added. The supernatant and the phenol/chloroform/isoamyl alcohol were homogenized using Benchmark BeadBlaster 24. The mixture was centrifuged and 250 ml of supernatant was then transferred to a new tube, to which 25 ml of 3 M pre-chilled sodium acetate (pH 5.2) and 300 µl isopropanol (Sigma-Aldrich, USA) were added to precipitate the DNA. After the resultant supernatant was discarded, 500 ml of 70% ethanol was added to wash the DNA and the tubes were centrifuged. The supernatant was again discarded, and the tubes were dried on a heat block incubator at 60°C for 30 minutes. The DNA was then eluted by the addition of 200 ml of 1xTE buffer (pH 8.0).

Maternal vaginal swabs

The collected vaginal swabs were first vortexed for 5 minutes to resuspend the cells, and 500 µl of the

aliquot from each swab was transferred to a new tube. To lyse the cells, 50 µl of lysozyme (10 mg/ml), 35 ml (140 U) of mutanolysin, 1 ml of lysostaphin and 50 ml of TE50 buffer (10 mM Tris-HCL and 50 mM EDTA, pH 8.0) were added to the tube, which was then incubated for 1 hour at 37°C. The mixture was transferred to a tube containing 0.05 g of glass beads (0.1 mm diameter), and the cells were agitated in the Benchmark BeadBlaster 24. The DNA was extracted from the resulting lysate using the QIAamp DNA Mini Kit, following the manufacturer's protocol, and eluted by the addition of 50 µl of Buffer AE.

Breast milk samples

First, 5 ml of each breast milk sample was centrifuged at 5,000x g for 20 minutes at 4°C. After centrifugation, the top creamy layer and the remaining supernatant layer were removed and the pellet was washed twice with 1 ml of 1xPBS.

After washing, 500 µl of proteinase K, 8 µl of RNAse A stock solution (100 mg/ml), and 200 µl of Buffer AL from the QIAamp DNA Mini Kit were added to the pellet. The mixture was mixed after the addition of each reagent by pulse-vortexing for 15 seconds. After incubation at 56°C for 10 minutes, 1 ml of ethanol was added and the mixture was mixed again by pulse-vortexing for 15 seconds. The DNA was extracted from the resulting lysate using the QIAamp DNA Mini Kit, following the manufacturer's protocol, and eluted by the addition of 50 µl of Buffer AE.

Next-generation DNA sequencing

Quantification of double-stranded DNA

The concentration of double-stranded DNA extracted was measured using the Quanti-it™ PicoGreen® kit (Invitrogen, USA). After quantification, each DNA sample was normalized to approximately 12.5 ng for polymerase chain reaction (PCR).

16 S rRNA amplicon production and purification

The KAPA HiFi™ PCR Kit (Kapa Biosystems, USA) was used in the PCR for 16 S rRNA DNA amplicon production. The reaction mixture for each DNA sample included 12.5 µl of 2x KAPA HiFiHotStart Ready Mix, 0.5 µl each of forward and reverse primers, and 11.5 µl of the normalized DNA sample. The PCR was done in a thermocycler as follows: initial denaturation at 95°C for 3 minutes, followed by 25 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension cycle at 72°C for 5 minutes. After the PCR, the products were purified using Agencourt®AMPure®XP beads (Beckman Coulter, USA) and resuspended in 50 µl of 10 mMTris buffer (pH 8.5).

Addition of indices and adapters in Index PCR and purification of products

The reaction mixture for each DNA sample included 12.5 µl of 2x KAPA HiFiHotStart Ready Mix, 5 µl each of i7 and i5 primers (Illumina, USA), and 5 µl of the DNA amplicons produced in the previous PCR. The PCR was performed as follows: initial denaturation at 95°C for 3 minutes, followed by eight cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. The libraries produced were purified again using Agencourt®AMPure®XP beads and resuspended in 25 µl of 10 mM Tris buffer (pH 8.5).

Library normalization, pooling, and re-quantification by Quantitative PCR (qPCR)

The library concentration for each sample was measured with the Quant-it™PicoGreen® kit (Invitrogen, Carlsbad, CA, USA). Each library was then normalized to the required concentration with 10 mM Tris buffer (pH 8.5), and 5 µl of each normalized library was pooled into a tube. The resulting pooled amplicon library (PAL) was then re-quantified via qPCR using the KAPA Library Quantification Kit (Kapa Biosystems, USA) following the manufacturer's protocol.

Denatur ationand dilution of library and PhiX Control

A 1:1 volume ration of 0.2 N sodium hydroxide (NaOH) and PAL was used to denature the DNA. The solution was vortexed briefly and incubated at

room temperature for 5 minutes. Then, a 100x dilution of pre-chilled hybridization buffer (HT1) was added. The resultant denatured amplicon library (DAL) was diluted again with pre-chilled HT1 to a pre-defined concentration. The PhiX control was also denatured and diluted following the above procedure and spiked in at 20% to form the final combined library (the DAL and PhiX control libraries). The combined library was heat-shocked and cold-shocked before being placed in an Illumina© Miseq Desktop Sequencer (Illumina, USA) using the Miseq Reagent v2 (500 cycles) run cartridge (Illumina, USA).

Analysis of DNA sequence data

Quantitative Insights Into Microbial Ecology (QIIME) in 16 S rRNA DNA amplicon data analysis

The 16 S rRNA DNA sequence data obtained were analyzed with QIIME version 1.9.1.[61] In QIIME, the corresponding reverse and forward reads were joined, and the resultant paired reads were selected based on a Q-score of 25. Chimeric sequences were filtered out and removed using USEARCH v6.1.[62] The resultant sequences were then subjected to open-reference OTU picking, using Greengenes v13_8 as the reference database and a similarity threshold of 97%. The OTUs were then mapped using a taxa summary to further interpret the bacterial profiles of the samples. The bacterial genus data were further analyzed to compare the mothers' and children's profiles. The mean percentage of shared OTUs between the mothers' and children's samples in each children's age group was also calculated. Using the Canoco5 software package (Microcomputer Power Co, Ithaca, USA), a principal component analysis (PCA) was performed on the square-root of Bray-Curtis distances based on the relative abundance of bacterial genus data.

Correlation index

A correlation index, comprising a summation of correlation values (r) for commensals and potential pathogens was used to quantify the degree of correlation. A zero-value correlation index implies no net correlation between any of the commensals and pathogens. The maximum positive correlation index is 168 (15 commensals x 14 pathogens x 0.8), whereas the highest negative correlation index is −84 (15 commensals x 14 pathogens x [−0.4]). The potential pathogens included were *Neisseria, Pseudomonas, Bacteroides fragilis, Campylobacter ureolyticus, Clostridium difficile, Clostridium perfringens, Haemophilus influenzae, Haemophilus parainfluenzae, Klebsiella, Prevotella melaninogenica, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus agalactiae*, and *Streptococcus anginosus*. The commensal species included were *Akkermansia, Bifidobacterium, Blautia, Enterococci, Faecalibacteria, Lactobacilli, Ruminococci, Escherichia coli*, other *Bacilli*, other *Bacteroides*, other *Campylobacter*, other *Clostridia*, other *Prevotella*, other *Staphylococci*, and other *Streptococci*.

Fecal water preparation

A mixture of 0.01 M Phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, USA) and 1% Bovine Serum Albumin (BSA) in PBS solution was freshly prepared. Approximately 1 g of the freshly collected fecal sample was mixed with twice the volume of the prepared PMSF-BSA-PBS solution by vortexing. After centrifugation at 4,000 g for 5 minutes, the supernatant was transferred into a new tube and then centrifuged at 4,000 g for 10 minutes. The supernatant was transferred into another new tube and stored at −80°C for further analysis.

Fecal water cytokines analysis

Using the LUNARIS™ Human 11-Plex cytokine kit (AYOXXA Biosystems, Austria), the levels of IL-1β, IL-2, IL-4, Il-5, IL-6, IL-8, IL-10, IL-12, IFN-γ, TNF-α, and GM-CSF were measured. The thawed and clear supernatant of fecal water was diluted in assay diluent 1. The diluted standards, diluted samples, and blanks (replicates) were first prepared in a 384-well microplate. All of them were transferred into a LUNARIS™ BioChip and prepared according to the manufacturer's protocol. Fluorescence images were captured with a fluorescence microscope (Zeiss Axio Imager M2, Zeiss, Germany), and quantification was performed using the LUNARIS™ analysis suite included in

the LUNARIS™ accessory kit (AYOXXA Biosystems, Austria).

Bile acids analysis

Approximately 200 mg of fecal aliquot was mixed with three times the volume of feces extraction buffer (20 mM phosphate buffer and ethanol) (Sigma-Aldrich, USA). The homogenized samples were sonicated at 70 W and 0°C for 5 minutes after shaking at 0° C for 30 minutes. After two centrifugations, the supernatant was transferred into a new reaction tube. Then, 10 µl of the extracted fecal samples were taken for bile acids analysis using Biocrates® Bile Acids kit (Biocrates Life Sciences AG, Austria), following the manufacturer's protocol. The analysis was conducted using the Agilent 1290 Infinity high-performance liquid chromatography system (Agilent Technologies, Germany) coupled to the AB SCIEX QTrap 5500 (mass spectrometry) (AB SCIEX Pte. Ltd, USA). The chromatograms were integrated using MultiQuant 3.0 software SCIEX (AB SCIEX Pte. Ltd, USA). The following bile acids were measured: Chenodeoxycholic acid (CDCA), colic acid (CA); ursodeoxycholic acid (UDCA), deoxycholic acid (DCA), lithocholic acid (LCA); glycochenodeoxycholic acid (GCDCA), taurochenodeoxycholic acid (TCDCA), taurocholic acid (TCA), glycocholic acid (GCA), glycodeoxycholic acid (GDCA), glycolithocholic acid (GLCA), hyodeoxycholic acid (HDCA), tauroursodeoxycholic acid (TUDCA), glycoursodeoxycholic acid (GUDCA), taurodeoxycholic acid (TDCA), alpha- and beta-tauromuricholic acid (TMCA [a + b]) taurolithocholic acid (TLCA), primary bile acids (sum of CDCA and CA) and secondary bile acids (sum of DCA, LCA, and UDCA).

Weaning foods questionnaire analysis

The food items were categorized into ten groups: complex carbohydrates, vegetables, legumes and soy, fruits, meats, dairy products, snacks, beverages, fast foods and seasonings (Text S1). The recorded frequencies and serving sizes of each food item consumed by the weaned children were calculated as consumption per day and further converted into the total amount of consumption in grams in a day.

Statistical analysis

All statistical analyses and data visualization were performed using GraphPad Prism 8 (GraphPad Software Inc., USA) and R 3.5.2 software (RStudio, Inc., USA). A permutational multivariate analysis of variance (PERMANOVA) was performed using the pairwiseAdonis R package.[63] The *p* values were corrected by post-hoc Bonferroni multiple comparisons. The two-way analysis of variance (ANOVA) and the post-hoc Bonferroni multiple comparisons test were performed on the data of the relative abundance of bacterial genera, fecal water cytokines and bile acids to check the significant differences in the distribution of bacterial abundances (> 1% of total OTUs), cytokines, and bile acids in the samples between children's age groups and between children's and mothers' samples. The Kruskal-Wallis test and the post-hoc Dunn's multiple comparisons test were performed to check for significant differences in individual food groups between children of different age groups after weaning. The Spearman non-parametric correlation test was also used to identify correlations between the OTUs of known gut commensals species and potential pathogens and between 1% of bacterial genera and the different food groups in weight using microbiome R package[64] and GraphPad Prism 8.

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Author contributions

Y-K. L and W. W. T. K wrote the main manuscript text. W. W. T. K, T. Y. S and S. K carried out the experimental work. W. W. T. K prepared the data analysis, performed data visualization and statistical analysis. Y-K. L and E. S. R led the study design, overall research collaboration, and coordination. S. S and J. N edited the manuscript. All authors reviewed the manuscript.

Availability of data and materials

All the data generated and the details methods in this paper were provided in the information, protocol exchange (2019) DOI: 10.21203/rs.3.pex-742/v1 and EBI repository (accession no: PRJEB34323). Materials and data should be addressed to Yuan-Kun Lee (micleeyk@nus.edu.sg).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Ethics approval and consent to participate

The Universitas Gadjah Mada's review board approved this study. All individuals before participation obtained informed consent and all experiments were performed by approved guidelines and regulations.

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Gut microbiome of pre-adolescent children of two ethnicities residing in three distant cities

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Recent studies have realized the link between gut microbiota and human health and diseases. The question of diet, environment or gene is the determining factor for dominant microbiota and microbiota profile has not been fully resolved, for these comparative studies have been performed on populations of different ethnicities and in short-term intervention studies. Here, the Southern Chinese populations are compared, specifically the children of Guangzhou City (China), Penang City (west coast Malaysia) and Kelantan City (east coast Malaysia). These Chinese people have similar ancestry thus it would allow us to delineate the effect of diet and ethnicity on gut microbiota composition. For comparison, the Penang and Kelantan Malay children were also included. The results revealed that differences in microbiota genera within an ethnicity in different cities was due to differences in food type. Sharing the similar diet but different ethnicity in a city or different cities and living environment showed similar gut microbiota. The major gut microbiota (more than 1% total Operational Taxonomy Units, OTUs) of the children population are largely determined by diet but not ethnicity, environment, and lifestyle. Elucidating the link between diet and microbiota would facilitate the development of strategies to improve human health at a younger age.

The gastrointestinal tract contains the greatest number of colonized microbes, mostly anaerobes, estimated to cover 70 percent of all the microbes in the human body[1]. Existing at such high concentrations in our body, it has been found that these gut microbes play important roles in modulating our health and diseases[2–4]. Thus, it is most relevant to understand factors determining gut microbiota profile.

Diet is an important factor determining the dominant gut microbiota[1,5,6]. A western diet consists of high fat, refined carbohydrates, and low fiber, resulting in European having high *Bacteroides* abundance and an under-represented *Prevotella* genus[7]. Whereas the Africans have high levels of *Prevotella*, resulting from the high fiber content in their diet[7]. This is due to the *Prevotella* genus containing many species with high fiber degrading potential[8]. However, in this and other studies on long-term habitual dietary consumption in distant regions and close vicinity of different ethnicity[9–11], the contribution of human genes, living environment and lifestyle in the microbiota profile could not be verified.

Many intervention studies had demonstrated that short-term diet changes[12] and seasonal variation in dietary habit[13] can affect the gut microbiota, but the magnitude of change did not result in a permanent alteration of enterotype. They also could not verify the factor(s) that determine the stability of the microbiome. A recent publication reported that individuals with distinct ancestral origins who shared a relatively common environment showed significant similarities in the compositions of the microbiomes[14].

In our project, we focused on three Asian cities, Guangzhou China, Penang West coast Malaysia, and Kelantan East coast Malaysia. The two Malaysian cities are populated by Chinese of Southern China ancestry. All three cities also have the same tropical climate and are all surrounded by sea (food source). Whilst all three population

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consume Chinese food, Malaysia Chinese also consume the local diet (Malay foods) and local ingredients. For instance, the Peranakans of Penang consume Chinese-Malay fusion food.

This study aims to compare the Chinese (CN) population of Penang (PN), Kelantan (KL), and Guangzhou (GZ) based on their microbiota and diet. Having the same ancestors, it would be interesting to find out if their gut microbiota will be similar due to their race or will it segregate based on their different locations and dietary habits. The proportion of Chinese in the two Malaysian cities is different. The percentage of Malays (ML) and Chinese in Penang are 41.6% and 41.5% respectively, which is approximately equal. As for Kelantan, the percentage of Malays and Chinese in the region is 92.3% and 3.2% respectively, making the Chinese population a minority race in Kelantan[15]. The difference in the proportion of Chinese in the two cities will also result in the difference in the level of influence in the food culture. Hence, the food consumed by the Chinese in Kelantan would have a higher level of influence by the Malay culture than that in Penang. If the hypothesis that diet is the most important influence on gut microbiota, then based on the level of influence in food culture, the gut microbiota of the Penang Chinese should be more similar to that of the Guangzhou Chinese than that of the Kelantan Chinese. The highest difference in gut microbiota should be the highest between that of Guangzhou Chinese and Kelantan Chinese. Besides, Penang is an urban modern city, while Kelantan is rural and largely agricultural. Children were chosen in this study, for they consumed more uniform and traceable home cooked food, and at a critical age whereby the microbiota profile has established to that of the adult-like[16] and may determine later life health status.

Other than comparing the Chinese population, the Penang and Kelantan Malay samples were also collected for comparison analysis. This is to investigate if the gut microbiota of the Penang and Kelantan Chinese would be similar to that of other Chinese populations from another country (China) or to the Malays who reside in the same city sharing many food types, living environment, and lifestyle.

Results

Effect of ethnicity on microbiota. To access the impact of ethnicity on gut microbiota, (1) two different ethnicities; namely, the Malay and Southern Chinese living in Penang and Kelantan, and (2) the Chinese children from Guangzhou, Penang, and Kelantan were compared.

Firstly, the unweighted Principle Coordinates Analysis (PCoA) plot of the Chinese population showed a separation between the Guangzhou and the Kelantan children (Fig. 1) at a p-value of 0.001 statistically and comparatively high R-value of 0.517 (Supplementary Table S1). There was also a separation between the Chinese children of Guangzhou and Penang ($p = 0.001$, $R = 0.328$), as well as between the Kelantan and Penang ($p = 0.001$, $R = 0.380$). Similarly, a separation for the Malay population between Kelantan and Penang children ($p = 0.001$, $R = 0.249$) was observed. The separation is significant by the statistical analysis of Analysis of Similarities (ANOSIM) and can also be clearly seen in the PCoA plots (Fig. 1a,c).

As for the weighted PCoA plots (Fig. 1b,d), there was a separation between the Guangzhou and Kelantan Chinese ($p = 0.001$, $R = 0.260$) but there was no separation between the Guangzhou and Penang Chinese, as well as between the Penang and Kelantan Chinese. On the other hand, there was a separation between the Kelantan and Penang Malay children at p-value of 0.001 and R-value of 0.235 (Table S1).

The results of the unweighted PCoA plots show that the types of bacteria present in the microbiota of the children population from the separate locations are different from each other, as evident from the separation for the pairwise comparisons (Table S1). On the other hand, the results of the weighted PCoA plots show that the relative abundance of some bacterial types in the microbiota is significantly different between the Guangzhou and Kelantan Chinese children, as well as between the Penang and Kelantan Malay children. However, there was no difference in the relative bacterial abundances for the other two pairs.

The children of different ethnicities living in the same location were compared to each other (Fig. 2). For the Kelantan Malay and Chinese population, there was a separation between them in the unweighted PCoA plot ($p = 0.001$, $R = 0.164$) but no separation for the weighted PCoA plot (Fig. 2a,b and Supplementary Table S2). As for the Penang Malay and Chinese population, there was also no separation between them for both the unweighted and weighted PCoA plots (Fig. 2c,d and Table S2).

The results of the unweighted PCoA plots show that the types of bacteria present in the microbiota of Kelantan Chinese and Malay children are slightly different from each other as there was a separation in the unweighted PCoA plot. On the other hand, the microbiota of the Penang Chinese and Malay children are very similar as there was no separation between their microbiota. As for the weighted PCoA plots, the results show that the microbiota of the two different ethnicities in the same locations are very similar to each other as there was no separation for both the weighted PCoA plots.

In order to confirm the separation of sample clusters by the race factor, a Partitioning Around Medoids (PAM) Clustering approach and a Distance-based Redundancy Analysis (db-RDA) involving 175 identifiable genera were performed (Fig. 3a,b). It was interesting to observe that two optimized clusters (1 and 2) were found among each race in each city validated by a mean silhouette width of 0.356 (Fig. 3a). 68.16% and 31.84% of people belong to cluster 1 and 2 respectively. The total variation observed was 13.97% and the Monte Carlo test was significant ($p = 0.002$) at 499 permutations (Fig. 3b). Nevertheless, separation of Guangzhou Chinese from that of Penang and Kelantan Chinese could be clearly seen within the same cluster (Fig. 3, Supplementary Tables S3 and S4), particularly in Cluster 1. Moreover, the Chinese and Malay population of Penang and Kelantan within the same cluster are closer to each other and separation is not obvious as that of Guangzhou Chinese (Fig. 3, Supplementary Tables S3 and S4). Both methods suggested that the relative abundance of the bacteria reflects the distance between the cities and the types of bacteria determine the different clusters.

In comparing Operational Taxonomy Units (OTUs) above 1% of the total population of bacterial genera, total 17 bacterial genera were found significantly different between cluster 1 and 2 using the Mann-Whitney U test (Fig. 4 and Supplementary Table S5). Cluster 1 was dominated by 14 bacteria (Fig. 4a) and *Bacteroides* was the most abundant, whereas Cluster 2 was dominated by 3 bacteria and led by *Prevotella* (Fig. 4b). The ratio of both

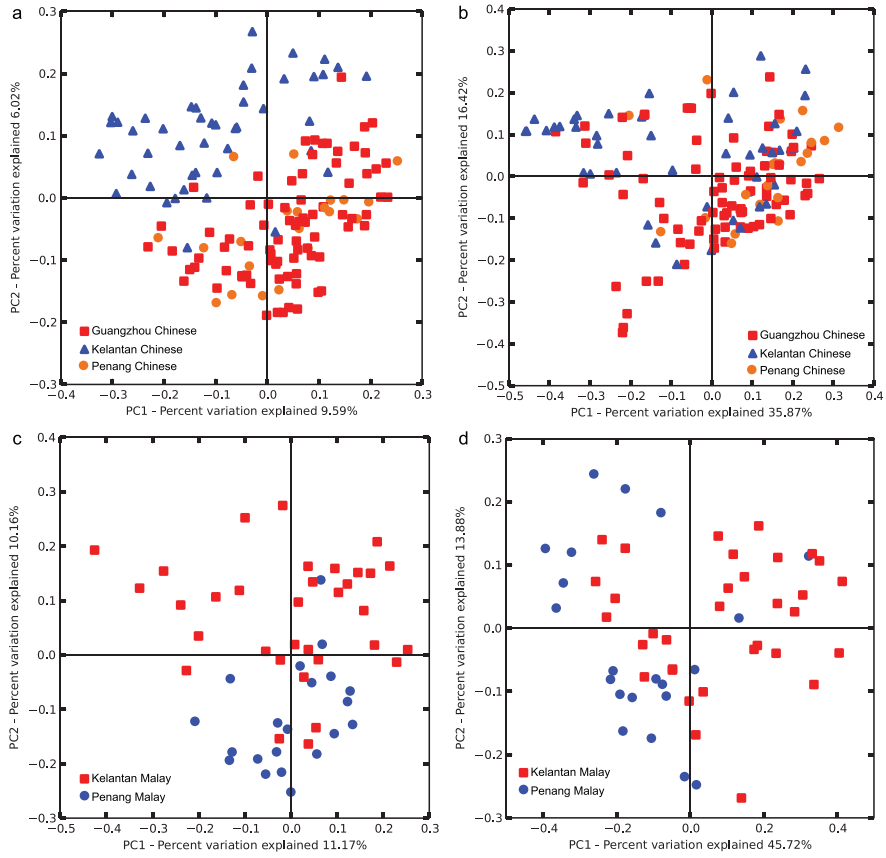

Figure 1. Unweighted and weighted UniFrac Principle Coordinates Analysis (PCoA) plots of microbiota among the same ethnicity. Two different races (Chinese and Malay) of children from Guangzhou, Kelantan, and Penang were compared against each other and visualized in the unweighted (left) and weighted (right) PCoA plots (**a**,**c** and **b**,**d**). The statistical analysis of ANOSIM was shown in Supplementary Table S1.

bacteria was found comparable within each cluster, but significantly different between two clusters across the different ethnicities and cities (Fig. 4c). Those major bacteria were found in the abundances order in a heatmap (d). *Bacteroides*, *Prevotella*, *Fecalibacterium*, and *Bifidobacterium* were the top 4 bacteria and the respective significant bacteria in pairs were found (Supplementary Tables S6–S8).

The predominant bacteria, with abundances above 1% of the total bacterial population in the two clusters are shown in Fig. 4d. Kruskal Wallis statistical test followed by Dunn's post-hoc multiple comparison tests were performed (Supplementary Tables S6–S8). In a pairwise comparison of each of the bacterium between the two clusters, in the same city and race, the abundance of *Bacteroides*, *Prevotella*, *Parabacteroides*, and *Streptococcus* was significantly different and may have contributed to the differentiation of the clusters (Supplementary Table S6). *Bacteroides, Parabacteroides*, and *Streptococcus* were predominant members of Cluster 1, whereas, *Prevotella* was the most abundant in Cluster 2.

Within the same city, the abundant of the four predominant bacteria (*Bacteroides, Prevotella, Oscillospira, Dorea, and Odoribacter*) were found to be significantly different between the two ethnicities (Supplementary Table S7). However, between the same ethnicity in the three different cities, numerous bacteria (18 out of the 27 of those above 1% of bacterial population) showed differences in their abundance, which suggests that geographical location-associated factor(s) but not the ethnicity is the major determinant of the bacterial abundance (Supplementary Table S8).

Effect of diet on microbiota. As diet could be a geolocation-associated causative factor in influencing gut microbiota, the effects of the diet were investigated. The correlation between the portion of 15 food types (complex carbohydrates enriched foods, oily foods, vegetables, refined sugars enriched foods, dairy products, fruits, meats, protein enriched plants, eggs, seafood, caffeinated drinks, biscuits/pastries/cakes, yoghurt, preserved foods and curry) and bacterial abundances at the genus level is shown in Fig. 5.

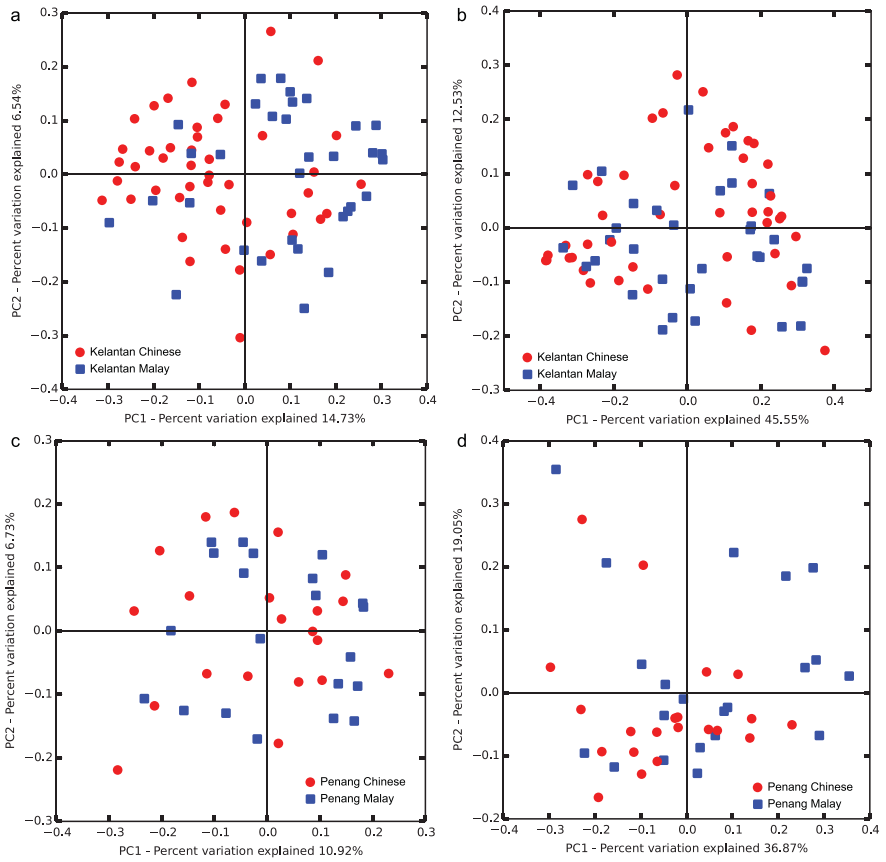

Figure 2. Unweighted and weighted UniFrac PCoA plots of microbiota between two ethnicities in the same location. Chinese and Malay population in two countries (Kelantan and Penang) comparison were separately plotted for race comparison in unweighted PCoA (**a**,**c**) and weighted (**b**,**d**). The statistical analysis of ANOSIM was shown in Supplementary Table S2.

Out of the 27 bacteria compared, while there are many significant correlations, there are four genera that stood out with high rho values ($\geq \pm 0.3$) among the 15 food types. The four genera are *Bacteroides, Fecalibacterium, Bifidobacterium*, and *Collinsella*. (Supplementary Table S9). Interestingly, all correlated with oily foods, vegetables, refined sugars enriched foods, fruits, meats, and curry and caffeinated drinks. Looking into the correlation between bacteria and the foods, *Bifidobacterium* and *Collinsella* are positively correlated with refined sugars enriched foods, while the latter is also positively correlated with fruits and curry foods. However, *Bacteroides*, *Faecalibacterium*, *Bifidobacterium*, and *Collinsella* are negatively correlated with caffeinated drinks, curry, oily foods, and Southeast Asian vegetables respectively.

Discussion

The results show one main point, which is that the difference in microbiota composition is mainly due to geographical location, be it locational dietary habit, ethnicity or environmental influence. When compared the different ethnicities living in the same location, there was no significant difference in the microbiota composition within the same cluster according to both PAM Clustering and dbRDA analysis. On the other hand, when children of same ethnicity living in separate locations, in particular, Chinese in Guangzhou and the two Malaysian cities within Cluster 1 were compared, there was a significant difference in the microbiota composition and abundance according to dbRDA analysis. Thus, the microbiota profile is not to any significant extent determined by ethnicity among the children. In a previous study on dichorionic triplet sets have shown that genetics do affect gut microbiota differences, but only at one month of age and after which, environmental factors play a larger role[17].

It is reasonable to assume that the subjects of separate locations are being exposed to different environments or lifestyles (cultures) and have access to different food types and hence will have different microbiota. A recent study done in mice has shown that mice in individually ventilated cages have different microbiota as compared to mice who live together in the same cage when they were all initially implanted with the same gut microbiota[18].

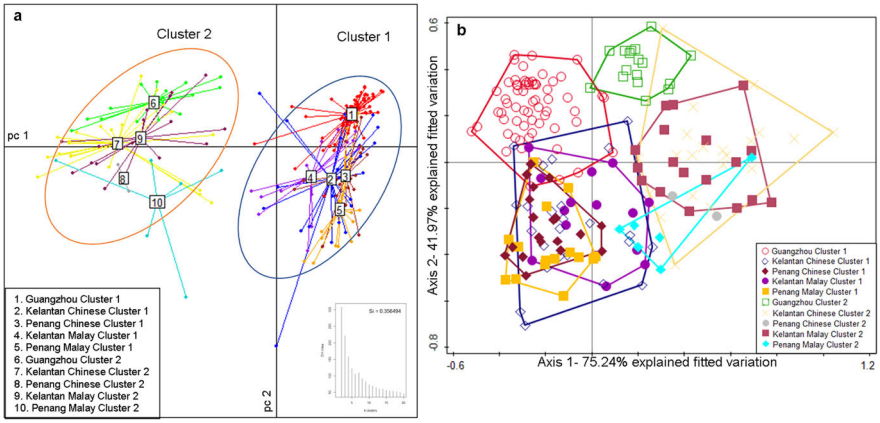

Figure 3. PCoA plot of PAM clustering using Jensen-Shannon distance (JSD) and db-RDA using Bray-Curtis distance comparing similarities between the two races from the three cities. Samples were revealed into two clusters clearly using PAM methods based on JSD distance matrix and bacterial genus abundances. (**a**) The two clusters were circled and within each cluster, the different races from the three cities were described in the different colors of centroid connectors. The Calinski-Harabasz (CH) index including the average silhouette (Si) coefficient was also shown. db-RDA plot was illustrated using Bray-Curtis distance and grouped into two clusters which estimated from PAM clustering method. (**b**) The percentage of variation explained for each axis is shown and the Monte Carlo test was significant at $p = 0.002$ and 499 permutations.

It was also shown that the microbiome similarity was found among the genetically unrelated individuals who shared household items[14]. This suggests that the environment can affect gut microbiota and those in the same environment will have similar gut microbiota types.

However, in our study among the children, microbiota profiles of an ethnicity (e.g. Chinese) resided in the same city (e.g. Guangzhou or Kelantan) are differentiated into the two clusters; while the same ethnicity in the same cluster (e.g. Chinese or Malay) living in distant cities (e.g. Penang and Kelantan) with different living environment and lifestyle (Penang being urban, while the Kelantan is rural) have similar microbiota profiles. This strongly suggested microbiota profile is to a little extent influenced by the living environment and further confirmed that ethnicity is not the major determining factor.

Despite different ethnicities, their food culture might have become more similar for the children in the same city (both Cluster 1 and 2). Malaysian cities are populated by Chinese and Malay, and thus most people consumed both Chinese and Malay types and fusion foods. Hence, it is no surprise that the microbiota of the Malaysian Chinese and Malay are very similar. On the other hand, the preferred food type is a personal choice and may explain the segregation in the microbiota between the clusters even within a city.

Among the three cities, higher consumption of refined sugar enriched foods, fruit, and curry led to a higher abundance of *Bifidobacterium* and *Collinsella* ($r \geq \pm 0.3$); whereas higher consumption of curry led to a higher abundance of *Faecalibacterium* and *Collinsella*. On the other hand, oily food and vegetable consumption was negatively associated with *Bifidobacterium* and *Collinsella* respectively. *Bacteroides, Faecalibacterium* and *Bifidobacterium* are the predominant bacteria in Cluster 1. The relative proportion of refined sugar, fruit, curry, oily food and vegetable, and perhaps not a single food item in the diet may have determined Cluster 1 profile. High fat consumption has been reported to associate with *Bacteroides*[7,19–21]. Cluster 2 is dominated by *Prevotella* and was not found to be strongly associated, positively or negatively with any of the food types in this study. Dietary fiber has been reported to promote *Prevotella* abundnace[7]. Nevertheless, *Prevotell*a is negatively associated with *Bacteroides*, as observed in this study and others[7,19–21].

A study done on Malaysian children of three ethnic groups namely Chinese, Malay, and aboriginal Orang Asli showed that the Chinese and Malay children share greater bacterial genetic lineages then when compared to the Orang Asli[22]. While one of the reasons could be the difference in dietary habit due to socioeconomic status, another reason they suggested was that the Chinese and Malay children were exposed to similar food types[22]. This agrees with the results of this study that diet is the main determinant of gut microbiota.

Interestingly, the predominant microbiota detected among Southern Han Chinese (Guangzhou in this study), are different from those found among the central and Northern Han Chinese (Wuxi, Chengdu, Zhengzhou and Harbin), where the gut microbiota of the youngsters was largely populated by *Phascolarctobacterium, Roseburia, Bacteroides, Blaustia, Faecalibacterium* and *Clostridium* in that order[23]. The gut microbiota profile of Guangzhou children is different from that of the central and Northern Chinese, but closer to that of the Southeast Asian.

Another interesting observation is that the Guangzhou children have almost no *Bifidobacterium* in their microbiota, as it is inversely proportional to the abundance of *Bacteroides*. This is of concern as many of the *Bifidobacterium* species are known to have health benefits. While *B. adolescentis* have been used for manufacturing of functional dairy products, *B. longum* lowers the risk of diarrhea and allergies in infants[24,25], and several

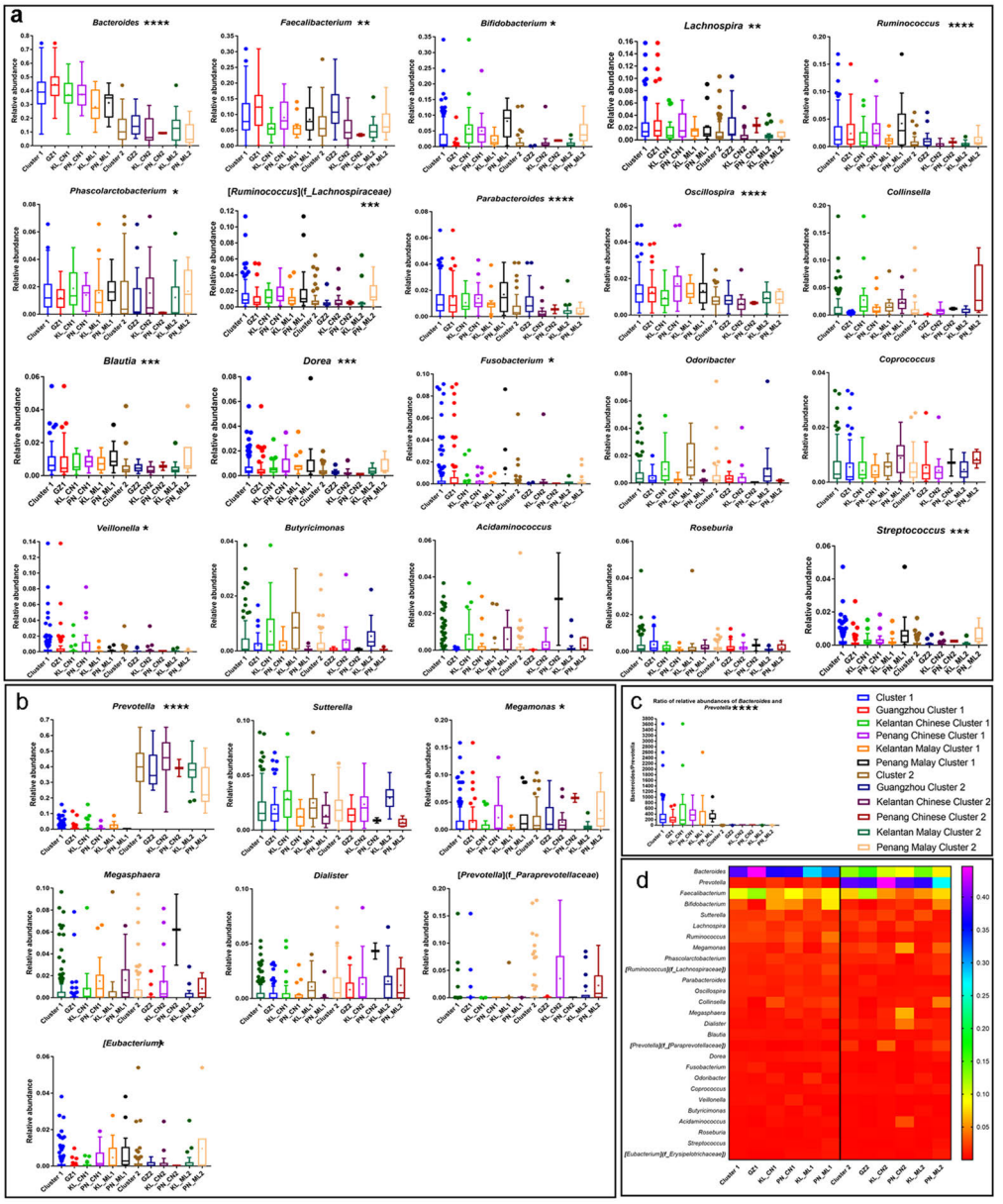

Figure 4. Relative abundance of major bacteria among the two ethnicities and the three different cities. Individual top 1% of the bacterial genera were plotted as box-plots. 20 bacteria were abundant in Cluster 1 (**a**) and 7 bacteria were rich in Cluster 2 (**b**). The ratio of Bacteroides and Prevotella abundances were compared (**c**). The symbol keys will be represented for (**a**–**c**) Overall major bacteria profile was shown as a heatmap (**d**). Relative abundances of OTU were illustrated as a spectrum of colors. The significant bacteria were marked with asterisks in a, b and c. Two-tailed ****$p < 0.0001$, ***$p \geq 0.0001$–<0.001, **$p \geq 0.001$–<0.01, *$p < 0.05$.

other *Bifidobacterium* species show benefits to gastrointestinal health as well. Hence, it would be relevant to investigate the gut microbiota health of Guangzhou children to verify if the lack of *Bifidobacterium* indeed resulted in a higher occurrence of gut-related diseases.

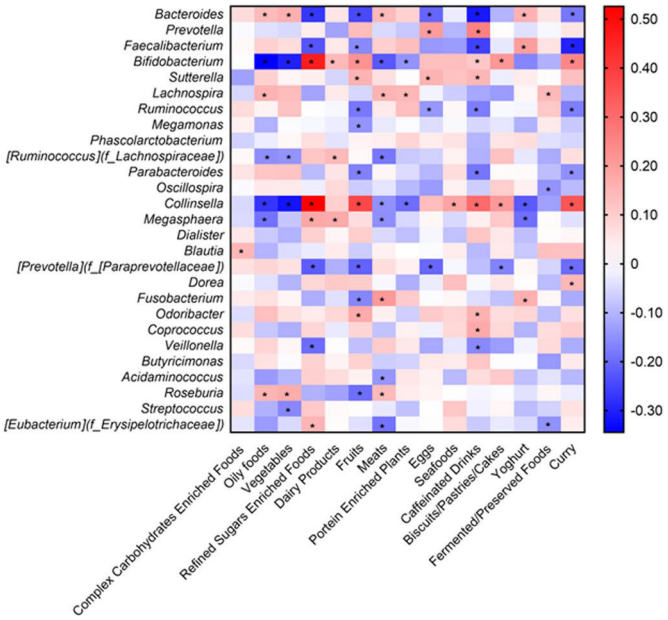

Figure 5. Correlation between the quantity of the various food types consumed in the fraction and bacterial abundances at the genus level. The food types and bacterial abundances were described in different axes. The Spearman rho (r) values were reflected as a color gradient and positively and negatively correlations are found in red and blue spectrums. The significant correlation is shown as asterisks * two-tailed $p < 0.05$.

Diet is a stronger influencing factor that can affect the changes in the relative abundance of gut microbes. From the correlation tables, it can be seen that the consumption of one food type can correlate with many genera and a single genus can also correlate with many food types. This shows that the food consumed is not exclusively used by one type of bacteria and the bacteria also do not exclusively feed on one type of food. The correlation between diet and microbiota is multidimensional, future metatranscriptomic and metabolomic studies would provide further understanding in their complex interrelation and may allow better modulation of microbiota through dietary intervention.

Conclusion

In summary, differences in the gut microbiota are largely due to dietary habit. Living environmental condition, and ethnicity are not determining factors for Southern Chinese and Southeast Asian children.

Methods

Ethics approval and consent to participate. The respective Institutional Review Board (IRB) at Guangzhou, Penang, Kelantan universities reviewed and approved the informed consent and the study protocols and other documents. The Joint Ethics Committee on Clinical Studies of School of Pharmaceutical Sciences, Universiti Sains Malaysia and Hospital Lam Wah Ee (JEC-SPS, USM & HLWE) (USM-HLWE/IEC/2014-0006) confirmed that the study was in compliance with the declaration of Helsinki and Malaysia Good Clinical Practice guidelines.

Study Design. A total of 201 local-born healthy children between 7 and 12 years old of both genders (male n = 110, female n = 91) were recruited at the respective schools from three cities: Guangzhou Southern Han Chinese (n = 81), Penang Southern Han Chinese (n = 21), Penang Malay (n = 21), Kelantan Southern Han Chinese (n = 45) and Kelantan Malay (n = 33). The participants who were healthy 7–12 years old children should not consume any pre/pro-biotics and antibiotics 2 weeks and 3 weeks prior respectively and have any recent illness that required to get medical attention last 3 months. All the participants had been informed and the consented documents were received from a parent upon the collection of samples.

Dietary intake questionnaires. Subjects were given the food frequency questionnaire (FFQ) requesting them to fill in the number of times they consume a specific type of food on a daily, weekly or monthly basis. We adopted the validated FFQ developed by the Singapore Health Promotion Board. The complete FFQs (Supplementary Text S12) and the raw data can be found. The questionnaire focuses on types, frequency, and the portion of foods and ingredients being consumed. We characterized the food items into 15 food types such as complex carbohydrates enriched foods, oily foods, vegetables, refined sugars enriched foods, dairy products, fruits, meats, protein enriched plants, eggs, seafood, caffeinated drinks, biscuits/pastries/cakes, yoghurt, preserved foods, and curry.

Collection of samples and DNA extraction. Approximately 10 g of stool was collected in 2 ml of RNA*later*® (Ambion, Inc., Texas, USA) and 0.2 ml of fecal homogenate was further washed with 1x Phosphate-buffered saline (PBS). The DNA extraction was done using the TIANamp Stool DNA kit (TIANGEN Biotech, Co., Ltd., Beijing, China) according to the manufacturer's protocol. Detail protocol (Supplementary Text 10) could be accessed.

Next-generation Sequencing. Once the concentration of the extracted double-stranded DNA had been measured using the Quant-iT™ Picrogreen® kit (Invitrogen, Inc., Carlsbad, USA), it was amplified with a Nextera® transposase sequences (details at S11 Text in supplementary information) and KAPA HiFi™ PCR kit (Roche life science, Inc., Indiana, USA). The PCR products were then purified by Agencourt® AMPure XP beads (Beckman Coulter, Inc., Fullerton, USA). In the index PCR, the reaction mixture was attached with the Illumina indexes (Illumina, Inc., San Diego, USA) using the KAPA HiFi™ PCR kit The DNA libraries produced were purified again using the Agencourt® AMPure XP beads. After the quantity of each library had been checked, it was normalised and pooled as Pooled Amplicon Library (PAL). Then, the PAL was then re-quantified with qPCR using the KAPA Library Quantification Kit (Roche life science, Inc., Indiana, USA).

The PAL and the internal control library (Illumina, Inc., San Diego, USA) were denatured with sodium hydroxide (NaOH) and diluted with a pre-chilled hybridization buffer (HT1) to the appropriate final concentration. The solution was sequenced on the Illumina® Miseq Desktop Sequencer (Illumina, Inc., San Diego, USA) The detailed protocol of the 16S rRNA sequencing preparation was described in Supplementary Text 11.

Data analysis. *Bioinformatics analysis.* The sequence data obtained were analyzed using QIIME (Quantitative Insights Into Microbial Ecology) version 1.9.1[26]. Using the QIIME the forward and reverse reads of the same sample were first joined. The paired reads were demultiplexed and quality filtered at Q-score of 25. Chimeric sequences were filtered out and Non-chimera were selected by USEARCH v6.1[27]. After which, using Greengenes v13.8 reference database, open-reference operational taxonomic units (OTUs) were picked out from the non-chimeric sequences at 97% similarity. The OTUs were summarized into taxonomy profiles of the bacteria in each sample. The bacterial genus data was provided. Beta diversity was calculated using the UniFrac distance matrices, generating the two- dimensional Principal Coordinate Analysis plots (PCoA). Weighted and unweighted UniFrac distance matrices were used to derive the beta diversity of the samples[28] which data could be accessed.

The relative abundance of genus data was filtered because the unassigned fraction was not considered in the calculation of clustering, distance-based redundancy analysis (db-RDA), the taxonomy classification and their statistical analysis. In total 175 genera were identified. A constrained distance-based redundancy analysis (db-RDA) was performed with the software CANOCO 5 (Microcomputer Power, USA). A relative abundance of bacterial genus level OTU data was applied and a classified triplot was drawn using Bray Curtis distance according to the manufacturer's instructions[29].

Clustering. Applying the approach of Enterotyping[30,31] using R version 3.5.1, the Jensen-Shannon distance (JSD) matrix was calculated based on the 175 relative abundances. The matrix was clustered by the Partitioning Around Medoids (PAM) algorithm using cluster package. Calinski-Harabasz (CH) index allowed estimation of the optimal number of clusters. The cluster number was validated using the individual and average silhouette coefficient (Si) and visualized by PCoA plots using the JSD matrix and adegraphics R package.

Statistical analysis. ANOSIM (Analysis of similarities) in QIIME was used to test for degree of separation between groups compared by using beta diversity Unifrac distance matrices as input. R-value and p-value shown in Table S1 and Table S2 were derived from Anosim test on the pairwise comparison based on unweighted and weighted Unifrac distance matrices. P-values < 0.01 are indicated with a * and shows significant separation.

For the bacteria at the genus level, only the top 1% highest abundance for each population were used for statistical analysis by GraphPad Prism 7 (GraphPad Software Inc., San Diego, USA). The non-parametric ANOVA; Kruskal-Wallis test, Mann-Whitney U test, and Multiple comparison post-hoc Dunn's test were performed to check for significant differences in the distribution of bacteria between the groups compared.

As for the diet data, the types of food consumed were classified into 15 different groups according to the FFQs, based on the frequency and portion of the food types consumed per month for every subject, and expressed as a fraction of total foods consumed. In addition, the bivariate Spearman rank correlation was done for correlation between the abundance of bacteria present and consumption of a food type.

A Permutational multivariate analysis of variance (PERMANOVA) test was performed for the JSD and the Bray Curtis distance matrices using pairwiseAdonis (pairwise comparison of vegan R package) followed by Bonferroni post-hoc test. For db-RDA model, Monte Carlo permutation test with 499 random permutations was also performed and pseudo F values, pseudo F statistics, and p values were generated.

Data Availability

The dataset generated or analysed during this study are available in the KNB repository, doi:10.5063/F12B8W9B and the rest of the data and materials are provided as the Supplementary Information Files.

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Author Contributions

W.W.T.K. prepared study design, was responsible for research coordination, samples processing, performed data analysis, generated figures and edited the manuscript. Y.Z. contributed to Guangzhou and Penang samples processing and drafted the manuscript. G.J.Y.G. contributed to Kelantan samples processing. M.S.W. co-ordinated Kelantan's samples. M.T.L. performed on Penang study collaboration, coordination and edited the manuscript. Y.Y.L. contributed to study design, Kelantan study collaboration and helped co-ordination. H.C. supported the Guangzhou samples preparation and coordination. Y.K.L. led the study design, overall research collaboration, coordination, and edited the manuscript. All authors read and approved the final manuscript.

Additional Information

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Gut Microbiome of a Multiethnic Community Possessed No Predominant Microbiota

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Abstract: With increasing globalisation, various diets from around the world are readily available in global cities. This study aimed to verify if multiethnic dietary habits destabilised the gut microbiome in response to frequent changes, leading to readily colonisation of exogenous microbes. This may have health implications. We profiled Singapore young adults of different ethnicities for dietary habits, faecal type, gut microbiome and cytokine levels. Subjects were challenged with *Lactobacillus casei*, and corresponding changes in microbiome and cytokines were evaluated. Here, we found that the majority of young adults had normal stool types (73% Bristol Scale Types 3 and 4) and faecal microbiome categorised into three clusters, irrespective of race and gender. Cluster 1 was dominated by *Bacteroides*, Cluster 2 by *Prevotella*, while Cluster 3 showed a marginal increase in *Blautia*, *Ruminococcaceae* and *Ruminococcus*, without a predominant microbiota. These youngsters in the three faecal microbiome clusters preferred Western high sugary beverages, Southeast Asian plant-rich diet and Asian/Western diets in rotation, respectively. Multiethnic dietary habits (Cluster 3) led to a gut microbiome without predominant microbiota yet demonstrated colonisation resistance to *Lactobacillus*. Although *Bacteroides* and *Prevotella* are reported to be health-promoting but also risk factors for some illnesses, Singapore-style dietary rotation habits may alleviate *Bacteroides* and *Prevotella* associated ill effects. Different immunological outcome was observed during consumption of the lactobacilli among the three microbiome clusters.

Keywords: cytokines; faecal microbiome; dietary habits; probiotics; immune response; multicultural dietary habit

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1. Introduction

Interactions between gut microbes and the human host are expected because of their lifelong association and proximity. Indeed, gut microbiome has been reported to play vital roles in the physiological functions and wellbeing of people [1,2]. *Bifidobacterium* and *Bacteroides* have been aligned with the maturation of host immunity in earlier life [3,4] correction of GI disorders associated with colitis [5], as well as behavioural and physiological abnormalities associated with neurodevelopment disorders [6]. *Prevotella* was reported as positively interfering in energy homeostasis and glucose control [7,8]. The first wave of gut microbes arrives mostly through vertical transmission from mother to child [9]. Upon introduction of solid foods, dietary habit has been demonstrated as the major determining

factor in gut microbiome composition, in studies comparing the microbiome of people across geographical regions and of different ethnicities [10–13]. Overall, high meat protein and fat, high sugar, non-resistant starch Western and Eastern Asian diets are associated with a *Bacteroides–Bifidobacterium* dominated gut microbiome [10–13], whereas the plant-, fiber- and carbohydrate- (high in resistant starch) rich diet of Southeast Asian and African type favours *Prevotella* in the gut microbiome [10–13]. These associations may be due to the provision of metabolisable nutrients and the resulting microenvironment. Establishment of the respective stable microbiome compositions facilitates colonisation resistance, in protecting the gut from being colonised by undesirable enteric pathogens arriving with foods [14].

Today, foods around the world are readily available in global cities, such as Singapore, where people consume foods of different origins in random rotation. It was the aim of this study to verify if Singapore's multiethnic dietary habit would destabilised gut microbiome in response to frequent changes, which may allow readily colonisation of exogenous microbes introduced orally. This may have health implications. In this study, the faecal microbiome and dietary habits of young Singaporeans were profiled and their associations were evaluated. The responses of each diet-determined microbiome cluster to an invading microbe (oral consumption of *Lactobacillus*) were assessed as an intervention model to study the gut microbiota structural stability and gut immune response to the invading microbe. Lactobacilli are bacteria with generally regarded as safe (GRAS) status and have been demonstrated to modulate the gut microbiome profile and immune functions as probiotics.

2. Materials and Methods

2.1. Study Design

A longitudinal randomised study was performed on 75 healthy young adults with an average age of 19.2 $\pm$ 1.9 years (18–30 years) of both genders (male: n = 30, female: n = 45), and ethnicities (Chinese: n = 61, Malay: n = 8, Indian: n = 2, Caucasian: n = 1, Filipino: n = 1, Korean: n = 1, Vietnamese: n = 1). Their BMI were 21.4 $\pm$ 2.9 at the baseline of the study. The subjects were recruited from a tertiary institution to minimise age, body weight and other confounding factors. They were instructed to maintain their dietary habit and lifestyle during the study. The total 42-days study included 14 days washout period (baseline; timepoint 1), 14 days ingestion period (two time points of 7 days apart each) and 14 days follow-up period (timepoint 4). At baseline and follow-up periods, the subjects abstained from the consumption of probiotic products. During the ingestion period, 100 mL of fermented milk containing the *Lactobacillus casei* (1×10^{10} CFU/mL) was provided every morning for 14 days. The dosage and period of consumption were chosen with reference to the Probiotics Fact Sheet for Health Professionals of the National Institutes of Health Office of Dietary Supplements 2020. The *Lactobacillus casei* was isolated from a local fermented food, identified by API 50 CHL kit (Biomerieux API), and thus is generally regarded as safe (Joint FAO/WHO Expert Committee on Food Additives). A *Lactobacillus* was chosen in this study for lactobacilli have been widely reported to modulate gut microbiome and host physiology (immunity). Milk was chosen as the culture and delivery medium, for dairy products were widely consumed among the subjects (consumed 2–12 times a week). Faecal samples were collected a total of four times at the end of each period. Foods frequency survey was collected at the baseline. Stool characteristics, frequency of defecation, water consumption and medications were recorded daily during the study. The protocol of the study is summarised in the following flow chart (Figure 1).

Inclusion criteria were 18–30 years old healthy adults who had normal BMI, no history of gastrointestinal disorder, not on long-term medication, no planning for overseas trip, able to drink the fermented milk with *Lactobacillus casei* everyday during the study period, able to abstain from any other fermented food products during the study period, and able to sign the informed written consent form. Exclusion criteria in the study were if the participants have allergy or intolerance to a special diet, using antibiotics, antimycotics, antidiarrheal or laxative medication in the 30 days prior to the study, lack of compliance

with the study protocol, or participating in any other studies within two weeks prior to the study.

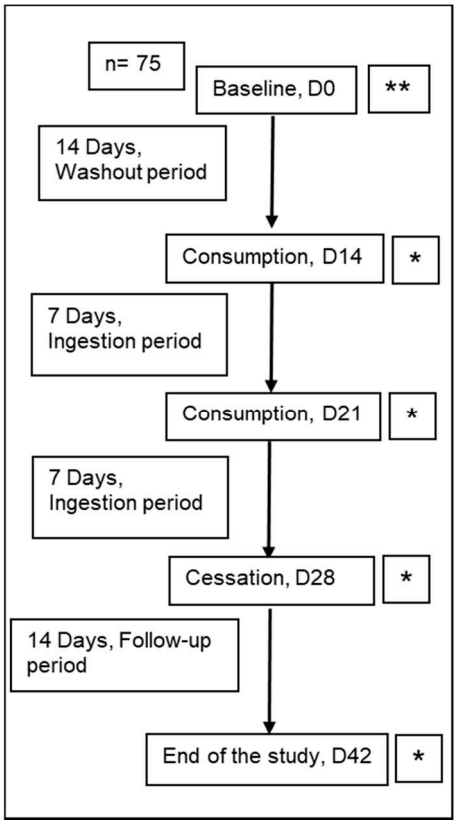

Figure 1. Flow chart of study design. Total 42 days of study, 14 days washout period, 14 days ingestion period and 14 days follow-up period. ** Food Frequency Questionnaire. * Faecal samples collection. D = Day, *n* = 75.

2.2. Food Frequency Questionnaire

All the subjects completed an in-house food frequency questionnaire (FFQ) (Supplementary Text S1) at baseline. Only 35 FFQ answers were able to be analyzed in this study due to the qualities of responses and missing data. The food items focused on are carbohydrate-rich foods (rice, noodles, cereals, bread, burger, French fries), protein-rich foods (meats, soy protein products), vegetables, fruits, nuts (peanuts, almonds, cashew, walnuts, macadamia, chestnuts), and beverages (coffee, tea, soft drinks, juices). The frequencies of food items that belonged to the same food category were summed up and the total frequency of food items was calculated per week.

2.3. Faecal Sample Collection and DNA Extraction

Approximately 1 g of faeces was collected and preserved in a collection tube containing 2 mL of RNAlater®® (Ambion Inc., Austin, TX, USA). A total of 75 faecal samples were collected. After weighing the samples, the faecal homogenate was diluted 10 times with RNAlater and 0.2 mL was washed with phosphate-buffered saline (PBS) (Axil Scientific Pte Ltd., Singapore, Singapore) which was later treated with Tris-SDS and TE-saturated phenol (Sigma-Aldrich, Cor., St. Louis, MO, USA) solution. After vigorously shaking, the

supernatant was mixed with phenol/chloroform/isoamyl alcohol (25:24:1) (Sigma-Aldrich, Cor., St. Louis, MO, USA) followed by homogenisation. Sodium acetate and isopropanol precipitated DNA was washed with 70% ethanol. Once dried, the pellet was eluted in TE buffer.

2.4. 16s rRNA DNA Sequencing

After DNA was quantified and calculated for polymerase chain reaction (PCR), the normalized 12.5 ng DNA was amplified with KAPA HiFi^{TM} HotStart ReadyMix kit (Roche life science, Inc., Wilmington, MA, USA) at v3 and v4 regions of the 16s rRNA gene. Amplified PCR products were purified with Agencourt$^{®®}$ AMPure XP beads (Beckman Coulter, Inc., Fullerton, CA, USA). The amplicons were added Nextera XT indices and adapter sequences. After two rounds of PCR, the DNA was purified again with the beads and eluted in Tris buffer. The library was quantified with Quanti-iT^{TM} PicoGreen$^{®®}$ dsDNA kit (Invitrogen, Inc., Carlsbad, FA, USA) and qualified with Agilent high sensitivity DNA kit (Agilent Technologies, Inc., Santa Clara, CA, USA) in Agilent 2100 bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). All the libraries were pooled and quantified with KAPA library quantification kit (Roche life science, Inc., Wilmington, MA, USA) in the ABI 7500 real-time PCR system (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The pooled library was denatured and diluted with NaOH and hybridisation buffer (HT1) (Illumina, Inc., San Diego, CA, USA) until the required titrated concentration. The denatured amplicon library was spiked with the PhiX control library (Illumina, Inc., San Diego, CA, USA) and run in Miseq sequencer (Illumina, Inc., San Diego, CA, USA).

2.5. Bioinformatics Analysis and Clustering

Quantitative insights into microbial ecology (QIIME) tool version 1.9.1 [15] was used for 16 s rRNA DNA sequence data. The selected paired reads were filtered and resulted in chimeric sequences by USEARCH v6.1 [16]. OTUs were picked by the open reference method and matched with 97% similarity sequences at Greengenes v13_8 database. Summarised taxa of relative abundance of OTUs revealed the bacterial genera in each sample. Alpha diversity indices were calculated using the OTU table referenced by the same database tree. Chao 1 and Shannon indices for alpha diversity were presented in this study. Unweighted and weighted UniFrac distance matrices for beta diversity were analysed using R package [17].

According to Entero-typing methods [18,19], the Jensen-Shannon distance (JSD) matrix was calculated using the relative abundance of bacterial genera data of Timepoint 2 in R v4. Clustering was performed by the partitioning around medoids (PAM) algorithm. The optimal number of clusters was estimated by Calinski-Harabasz (CH) index and validated by the individual and average silhouette coefficient (Si). Three classified clusters were named Cluster 1 (n = 31), Cluster 2 (n = 14) and Cluster 3 (n = 30). The assigned cluster to individual samples of Timepoint 2 was applied for further visualisation and categorisation of remaining samples.

A constrained Redundancy Analysis (RDA) based on the square root of Bray-Curtis distances (db-RDA) was performed using the relative abundance of genera data by the Canoco5 software package (Microcomputer Power Co, Ithaca, NY, USA). The principal coordinate analysis (PCoA) plots using unweighted and weighted UniFrac distances were visualised for beta diversity analysis by R v4.

2.6. Faecal Water Cytokines Analysis

One volume of faecal homogenate was treated with two volumes of 0.01 M Phenylmethyl-sulfonyl fluoride (PMSF) (Sigma-Aldrich, St. Louis, MO, USA), 1% Bovine Serum Albumin (BSA) and PBS solution. 10 µL of supernatant was diluted 1:3 ratio in assay diluent 1 of the LUNARIS^{TM} Human 11-Plex cytokine kit (AYOXXA Biosystems, Cologne, Germany) and loaded onto a LUNARIS^{TM} BioChip with its standards and blanks. The remaining procedures were followed as per the manufacturer's guide. Fluorescence from each well of

BioChip was read by a fluorescence microscope (Zeiss Axio Imager M2, Carl Zeiss Microscopy, Oberkochen, Germany) and quantified using the LUNARIS^{TM} analysis. 11 cytokines, namely, interleukins (IL) 1-β, -2, -4, -5, -6, -8, -10, -12, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon gamma (IFNγ) and tumour necrosis factor alpha (TNFα) were identified for 72 faecal water samples (Cluster 1: n = 30; Cluster 2: n = 14; Cluster 3: n = 28) for four time points. One sample was unable to analyse at Timepoint 2 of Cluster 3 due to the insufficient amount. The concentration of each sample in pg/mL was calculated and converted to pg in g of wet weight of faeces in the collection tube by multiplied the dilution factor. The lower limit of quantification (LLOQ) was used as a cut-off.

2.7. Statistical Analysis

To compare the differences of the bacterial community in three clusters, ethnicities, genders and Bristol stool scales shown in the db-RDA plot, a permutational multivariate analysis of variance (PERMANOVA) was performed using the pairwiseAdonis R package [20]. Adjusted p values were derived from the post hoc Bonferroni multiple pairwise test and permutation test at 4999. All the data were checked for normality and subsequently analysed by the appropriate statistical methods. Individual major bacterial genera abundances (>1% of total OTU) and frequency of food items were analysed using a non-parametric Mann Whitney U test to compare the different clusters. To identify the correlations between abundances of the major bacteria and total frequency of food items, the non-parametric Spearman correlation test was performed using 35 same samples of bacteria and FFQ data (Cluster 1: n = 9; Cluster 2: n = 10; Cluster 3: n = 16). One-way analysis of variance (ANOVA) and Bonferroni multiple comparisons tests were applied for alpha diversity analysis to compare the clusters and time points. The comparison of bacteria abundances for four time points in each cluster was done by non-parametric matched test of Friedman and Nemenyi post hoc multiple pairwise comparison tests. The mixed-effects model or two-way repeated measures ANOVA followed by Bonferroni multiple comparisons tests were performed for the comparison of cytokines in different time points. All the statistical analyses were performed using GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Clustering of Basal GI Microbiome

The faecal microbiome of Singapore young adults was segregated into three clusters (Figure 2A, Supplementary Table S1). Cluster 1 was dominated by *Bacteroides,* which constitute 30% of the total operational taxonomical units (OTU), whereas *Bacteroides* in Cluster 2 and 3 constituted 8% and 12%, respectively, significantly lower than Cluster 1 (Supplementary Tables S2 and S3). The abundance of *Bacteroides* in Cluster 1 was traded off by a reduced fraction of *Ruminococaceae* and *Prevotella*, as compared to Clusters 2 and 3. Cluster 2 was dominated by *Prevotella* (21% of total OTUs). Whereas *Prevotella* in Cluster 1 and 3 constituted only <1% and 1% of total OTUs, *Bacteroides* and *Ruminococcus* were the lowest in Cluster 2 among the three clusters. Cluster 3 does not have a predominant microbiota, but with statistically higher proportion of *Blautia* (16% vs. Cluster 1, 12%, and Cluster 2, 12%), *Ruminococaceae* (8% vs. Cluster 1, 3%, and Cluster 2, 6%) and *Ruminococcus* (4% vs. Cluster 1, 1%, and Cluster 2, 1%). This showed a comparatively even distribution of major microbiota in Cluster 3.

3.2. Dietary Composition and Habit

In general, subjects in the three clusters consumed significantly different food types (Figure 2B), mainly sugary beverages, carbohydrate-rich and protein-rich foods, fruits, nuts, and vegetables (Figure 2B, Supplementary Tables S4 and S5). Cluster 1 consumed more sugary beverages (16.11 ± 6.82 per week), carbohydrate-rich (30.72 ± 12.19 per week) and protein-rich (35.94 ± 13.68 per week) foods mostly in the form of Western fast foods,

with a lower frequency of vegetables (5.22 ± 3.03 per week) as compared to Cluster 2 (carbohydrate 18.38 ± 7.60, protein 23.96 ± 14.53, vegetables 11.00 ± 4.57 per week).

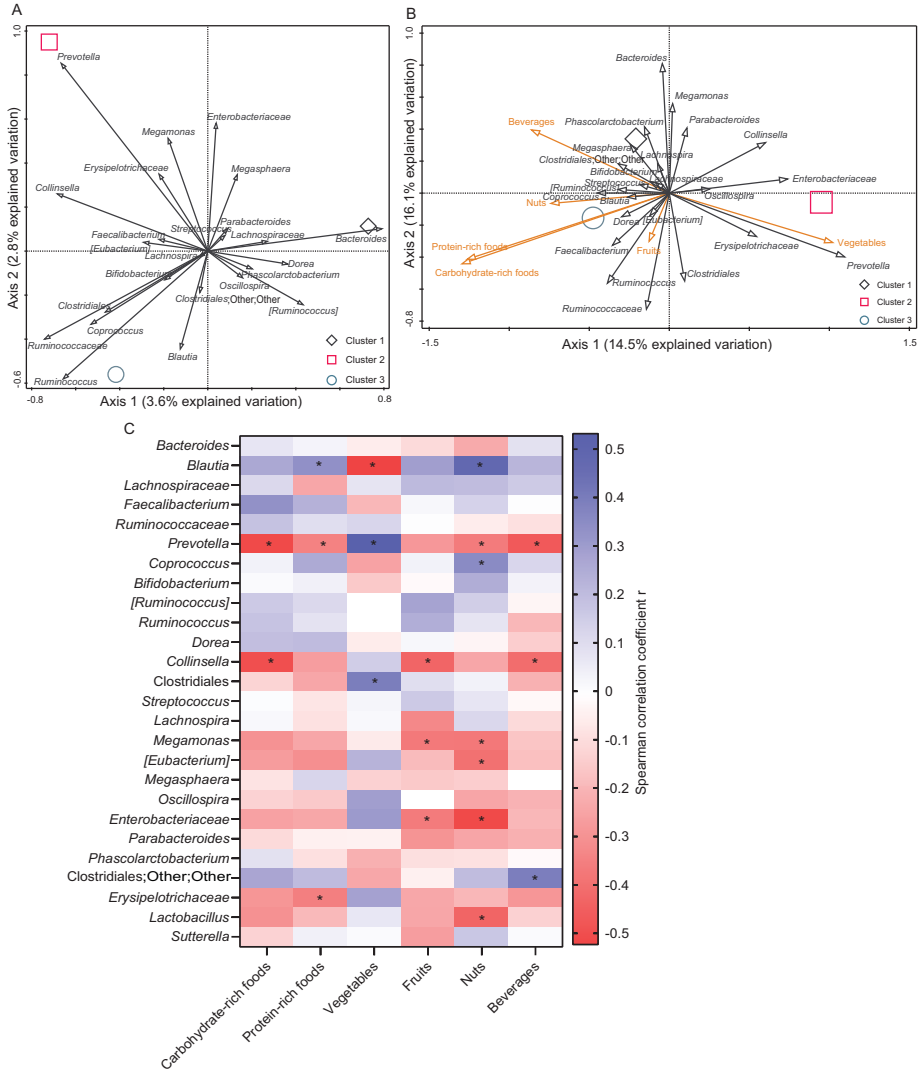

Figure 2. (**A**) Composition of major faecal microbiota and (**B**) distribution of dietary consumption and major faecal microbiota in three clusters at baseline. Species biplots of distance-based redundancy analysis (db-RDA) based on the square root Bray-Curtis distance matrix showed the distribution of faecal bacterial genera and the frequency of food items consumption per week in three clusters. The relative abundance of major (>1%) faecal bacterial genera (black arrows) and dietary consumption (orange arrows) shown here are concentrated with the respective arrow. The percentages of axes explain the compositional variation of the respective axis. The distances between each pair of clusters were tested for significance by permutational multivariate analysis of variance (PERMANOVA) and Bonferroni's multiple comparison pairwise test at 4999 permutations. Different symbols represent the types of clusters. (**C**) Correlation between the frequency of dietary consumption per week (*X*-axis) and major (>1% of total OTUs) faecal bacteria (*Y*-axis) at baseline. Spearman correlation coefficient r values in gradient scales were plotted and presented as a heatmap. The significantly different correlations (two-tailed $p < 0.05$) were marked with asterisks *.

The diet of Cluster 2 was typically of the plant-rich Southeast Asian type (vegetables), and Cluster 3 adopted the typical dietary habit of Singaporeans in interchanging Western and Asian foods. Overall, cluster 2 ate the lowest frequency of most food types (1 out of 6) among all clusters. However, it consumed more vegetables (11.00 ± 4.57 per week) as compared to Cluster 1 (vegetables 5.22 ± 3.03 per week) and Cluster 3 (vegetables 5.53 ± 2.57 per week). As indicated in Supplementary Table S5, Cluster 3 consumed the highest frequency of most food types (4 out of 6). Carbohydrate-rich and protein-rich foods, fruits and nuts were the most consumed foods in cluster 3 among all clusters, but the intermediate frequency of sugary beverages and vegetables compared to the other two clusters (Supplementary Tables S4 and S5). Thus, Cluster 3 is located in a different plane and between Clusters 1 and 2 in the square root of Bray Curtis distance-based Redundancy Analysis (db-RDA) (Figure 2B), although the adjusted *p*-value was not significantly different from Cluster 1 (Supplementary Table S6).

3.3. Correlation between Diet and Microbiota

In the heatmap correlation between major faecal bacteria and weekly frequency of food type consumption (Figure 2C), *Bacteroides* was found to correlate positively although not statistically significantly with carbohydrate-rich and protein-rich foods and sugary beverages. *Prevotella* was found to be positively and significantly correlated with vegetables, and negatively correlated with carbohydrate-rich, protein-rich foods, fruit, nuts, and sugary beverages, whereas *Blautia* positively correlated with carbohydrate-rich, protein-rich foods, fruits, nuts and sugary beverages; *Ruminococcaceae* positively correlated with carbohydrate-rich, protein-rich foods and vegetables; and *Ruminococcus* positively correlated with carbohydrate-rich, protein-rich foods, fruits and nuts. Other microbiota was positively or negatively correlated with the various food types.

3.4. Effects of Ethnicities, Genders and Types of Bristol Stool Scale in Association with GI Microbiome

As shown in Supplementary Figure S1 and Table S7, the major ethnic group in this study was Chinese ($n = 61$), whose faecal microbiome at baseline could be differentiated into three clusters. Malays ($n = 8$) Indians ($n = 2$) were randomly distributed among the three clusters. This might suggest that ethnicity was not a determining factor in the clustering of faecal microbiome among these Singapore youngsters. The rest of the ethnicities, namely Caucasian, Filipino, Korean and Vietnamese, comprised one subject each, and thus could not be accounted for in evaluating the effect of ethnicity.

The effect of gender on faecal microbiome is shown in Supplementary Figure S2 and Table S8 where the same gender was found to be significantly differentiated between different clusters at baseline and at all time points. However, both genders intermingled within the same cluster and thus clustering of faecal microbiota at baseline was not influenced by gender.

Among the seven Bristol stool scale types [21], type 3 and 4 stools are normal in stool shape and consistency, but type 1 is constipated and type 6 diarrhoetic stool. Generally, the microbiome in healthy stool (type 4) could be differentiated into clusters according to dietary habits (Supplementary Figure S3). In subjects with type 4 stool, microbiome from Cluster 1 was significantly different from that of Clusters 2 and 3 at baseline (Timepoint 1) and at Timepoint 3 during *Lactobacillus* consumption, and also significantly different from Cluster 3 at Timepoint 4 after the cessation of consumption (Supplementary Tables S9, S11 and S12). In stool type other than 4 (types 1, 2, 3, 5 and 6), the differences in microbiome distribution were not statistically obvious (Supplementary Tables S9–S12), as the subject numbers were few.

3.5. Biodiversity of GI Microbiome

Chao 1's and Shannon's indices estimated that the species richness and evenness between the clusters at baseline (Figure 3A,B) and between the time points in each cluster (Supplementary Figure S4A,B) were comparable statistically (Supplementary Tables S13

and S14), apart from, Timepoint 3 in Cluster 1, where there were more species than Timepoint 2 in the same cluster (adjusted p= 0.014) (Supplementary Table S14).

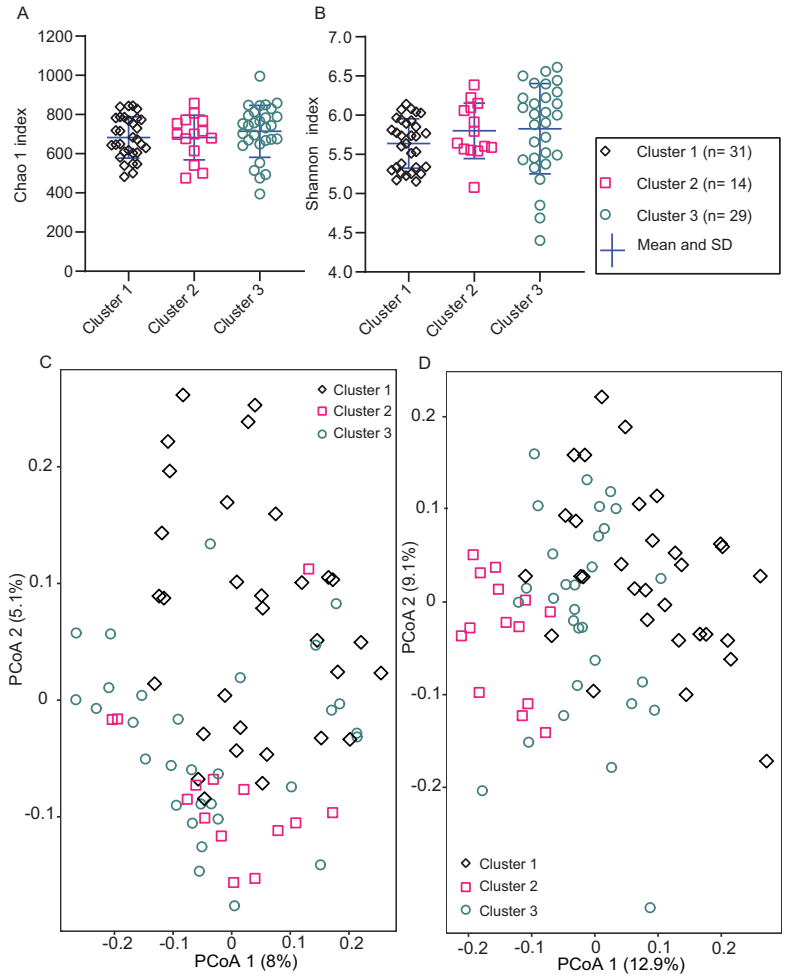

Figure 3. (**A**) Chao 1′s and (**B**) Shannon's indices of alpha diversity comparing three clusters at baseline. (**C**) Unweighted, (**D**) weighted Unifrac principal coordinates analysis (PCoA) for beta diversity comparing three clusters at baseline. Different symbols and colours represent different clusters. Means and SD of indices are presented. One-way analysis of variance (ANOVA) followed by Bonferroni multiple comparisons tests were applied and the indices were not significantly different between the clusters at baseline of each cluster. The distances between each cluster were tested for significant difference by permutational multivariate analysis of variance (PERMANOVA) and Bonferroni's multiple comparison pairwise test at 4999 permutations. Timepoint 1 = baseline; 14 days after washout, SD = standard deviation. Numbers of samples in parenthesis.

Both unweighted (Figure 3C) and weighted (Figure 3D) UniFrac distances were significantly different between the clusters at baseline (Supplementary Table S15) showing that the types and quantities of species were different between clusters at baseline. In Cluster 1, there was significant difference among all time points but no difference between Timepoint 3 and 4 in both unweighted and weighted UniFrac distances (Supplementary

Figure S5 and Table S15). In Cluster 2, each time point varied from the others in both unweighted and weighted UniFrac distances (Supplementary Figure S5 and Table S15). Unweighted and weighted UniFrac distances at all time points were different from each other in Cluster 3, but not between Timepoint 1 and 2 (Supplementary Figure S5 and Table S15).

3.6. Introduction of Exogenous Lactobacillus: Cluster 1

Most of the faecal major microbiome profile of Cluster 1 did not appear to alter significantly during the *Lactobacillus* administration (Figure 4A). On examination of the respective microbiota which constitutes >1% of total OTUs, only *Lactobacillus* showed a consistent trend ($p < 0.05$) of increase in abundance during the time of *Lactobacillus* consumption (Timepoints 2 and 3) (Supplementary Table S16). Its abundance returned to the baseline (Timepoint 1) on cessation of consumption (Timepoint 4) (Figure 4A).

As shown in Figure 4B and Supplementary Table S19, faecal water cytokines IL-1β, -2, -8, -12 and TNFα were detectable, while IL-4, -5, -6, -10, IFNγ, and GM-CSF were near to detection level across all time points in Cluster 1. Both cytokine IL-1β and -2 increased but IL-8, -12 and TNFα decreased at Timepoint 2. At Timepoint 3, IL-1β dropped nearly back to the level of Timepoint 1 but IL-2, -8, -12 and TNFα increased. Apart from IL-8, IL-1β, -2, -12 and TNFα increased among detectable cytokines at Timepoint 4. There was no significant difference in cytokine levels between genders at the same time points (Supplementary Figure S6A and Table S20).

3.7. Introduction of Exogenous Lactobacillus: Cluster 2

The faecal microbiome in Cluster 2 showed a significant reduction in the abundance of *Prevotella* at Timepoint 4 but not during *Lactobacillus* administration (Figure 5A and Supplementary Table S17). All other major faecal microbiota and *Lactobacillus* did not show significant variation at Timepoints 2, 3 and 4, as compared to their basal levels (Timepoint 1).

The basal (Timepoint 1) levels of faecal water cytokine IL-2, -12 and TNFα in Cluster 2 (Figure 5B) were 3–6 times higher than the basal levels in Cluster 1 (Supplementary Table S19). This implies higher lymphocytic and pro-inflammatory activities. Upon consumption of the lactic acid bacterium, the level of the pro-inflammatory IL-1β and -8 (Timepoint 3) and TNFα (Timepoint 2) increased, but the level of regulatory cytokine IL-2, -12 and TNFα (Timepoint 3) decreased. After cessation of *Lactobacillus* consumption, IL-8 decreased by 3.8 times but, IL-1β increased by 7.2 times. These showed that the *Lactobacillus* had a stronger immune-modulating effect on subjects in Cluster 2 than Cluster 1. No gender differences were found in cytokine levels at the same time point (Supplementary Figure S6B and Table S21).

3.8. Introduction of Exogenous Lactobacillus: Cluster 3

In Cluster 3, upon consumption of the *Lactobacillus*, some of the faecal microbiota profile was attenuated at Timepoint 4, in comparison with Timepoints 1, 2 and 3 (Figure 6A). The differentiation was due to a significant reduction in the abundance of *Bifidobacterium*, *Collinsella* and *Phascolarctobacterium* at Timepoint 4 (Supplementary Table S18). There was no difference in the abundances of other major microbiota, namely *Blautia, Prevotella* and *Bacteroides* over all time points. *Lactobacillus* abundance increased during consumption of the *Lactobacillus* (Timepoints 2 and 3) and returned to the basal level at Timepoint 4.

As shown in Figure 6B and Supplementary Table S19, the cytokine IL-8 decreased progressively from Timepoint 1 through Timepoint 4, whereas the level of IL- 2, -12 and TNFα decreased at Timepoint 2, but returned to the basal level at Timepoint 4. The level of IL-1β measured was variable across the time points, decreased at Timepoint 2 as compared to Timepoint 1, increased at Timepoint 3 and decreased again at Timepoint 4. The levels of cytokines were comparable between genders at the same time points (Supplementary Figure S6C and Table S22).

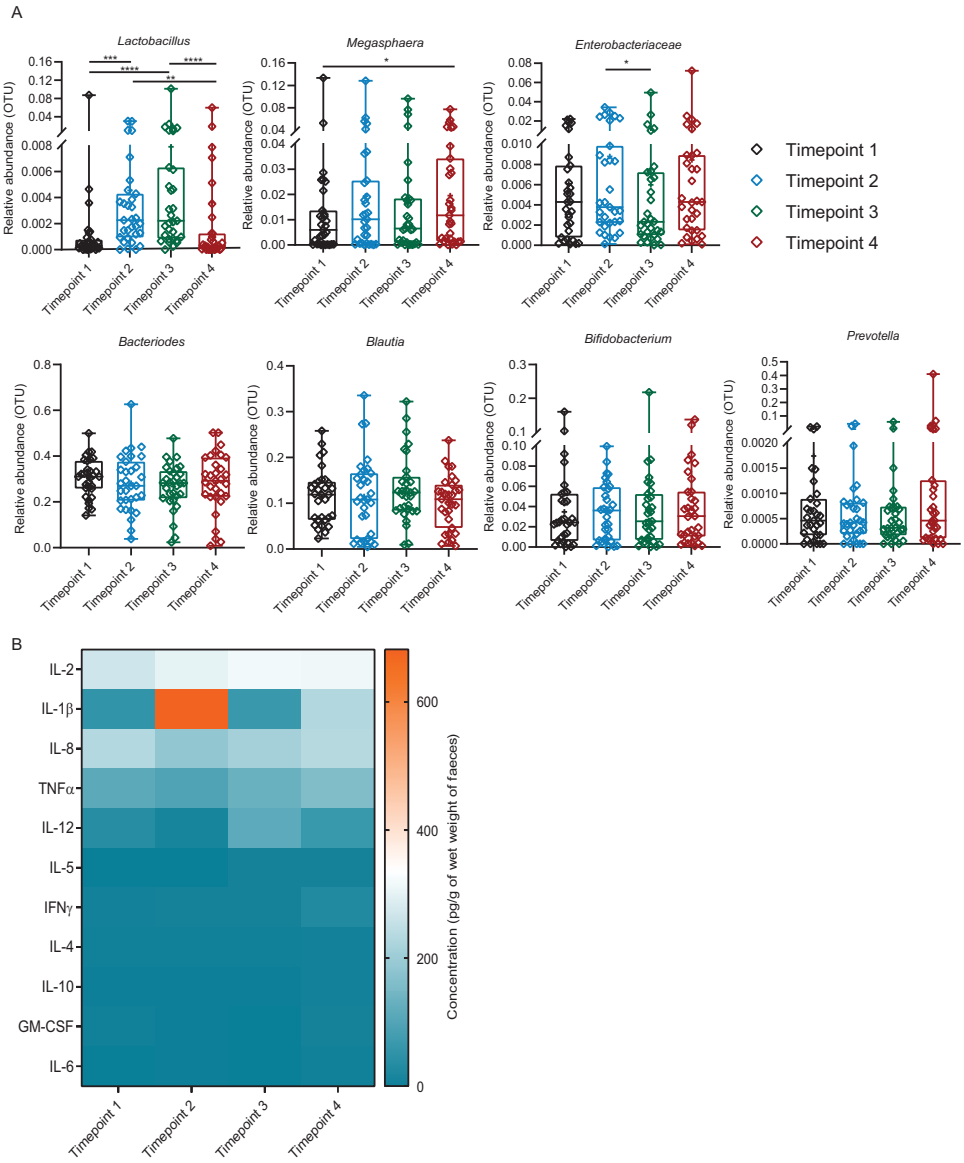

Figure 4. Profiles of faecal bacterial genera and cytokines of Cluster 1. (**A**) Comparison of relative abundance (OTUs) of seven major faecal bacterial genera of Cluster 1 across the time points. Different colours represent different time points. The bacteria which were significantly different between each time point (tested by Friedman rank-sum and post hoc Nemenyi multiple pairwise comparison tests) are presented as **** $p < 0.0001$, *** $p \geq 0.0001$–$p < 0.001$, ** $p \geq 0.001$–$p < 0.01$, * $p \geq 0.01$–0.05. (**B**) Comparison of concentration of faecal water cytokines (pg/g of wet weight of faeces) of Cluster 1 across the time points. Means of concentration are presented. No significant difference in the cytokine levels between the time points were found by two-way analysis of variance (ANOVA) followed by Bonferroni multiple comparisons tests. IL = Interleukin, TNF = Tumour necrosis factor, IFN = Interferon, GM-CSF = Granulocyte-macrophage-stimulating factor. Timepoint 1 = baseline; 14 days after washout, Timepoint 2 = first 7 days after ingestion, Timepoint 3 = second 7 days after ingestion, Timepoint 4 = follow-up; 14 days after non-ingestion. $n = 31$ for each time point.

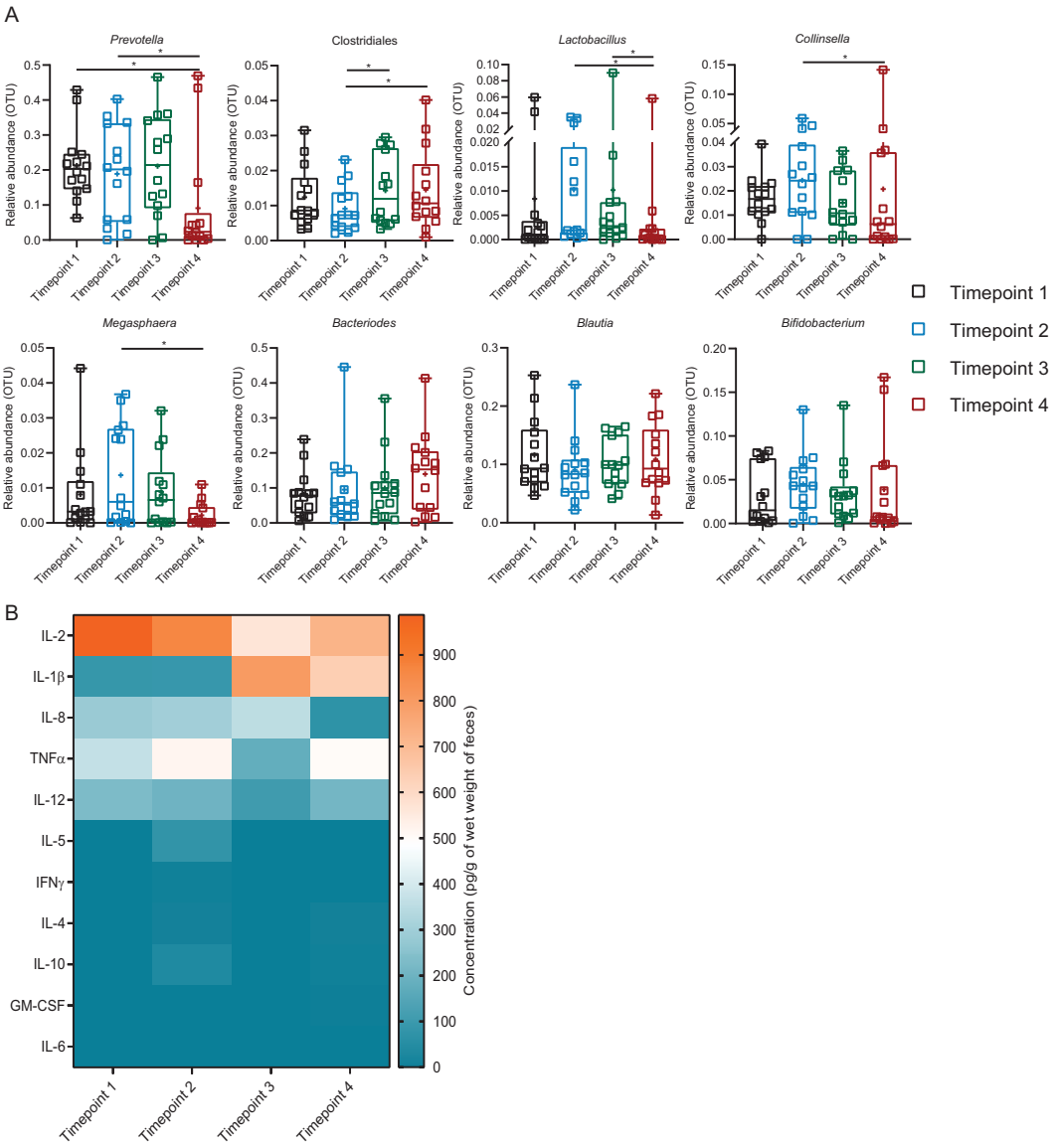

Figure 5. Profiles of faecal bacterial genera and cytokines of Cluster 2. (**A**) Comparison of relative abundance (OTUs) of eight major faecal bacterial genera of Cluster 2 across the time points. Different colours represent different time points. The bacteria which were significantly different between each time point (tested by Friedman rank-sum and post hoc Nemenyi multiple pairwise comparison tests) are presented as * $p \geq 0.01$–0.05. (**B**) Comparison of concentration of faecal water cytokines (pg/g of wet weight of faeces) of Cluster 2 across the time points. Means of concentration are presented. No significant difference in the cytokine levels between the time points were found by the two-way analysis of variance (ANOVA) followed by Bonferroni multiple comparisons tests. IL = Interleukin, TNF = Tumour necrosis factor, IFN = Interferon, GM-CSF = Granulocyte-macrophage-stimulating factor. Timepoint 1 = baseline; 14 days after washout, Timepoint 2 = first 7 days after ingestion, Timepoint 3 = second 7 days after ingestion, Timepoint 4 = follow-up; 14 days after non-ingestion. n = 14 for each time point.

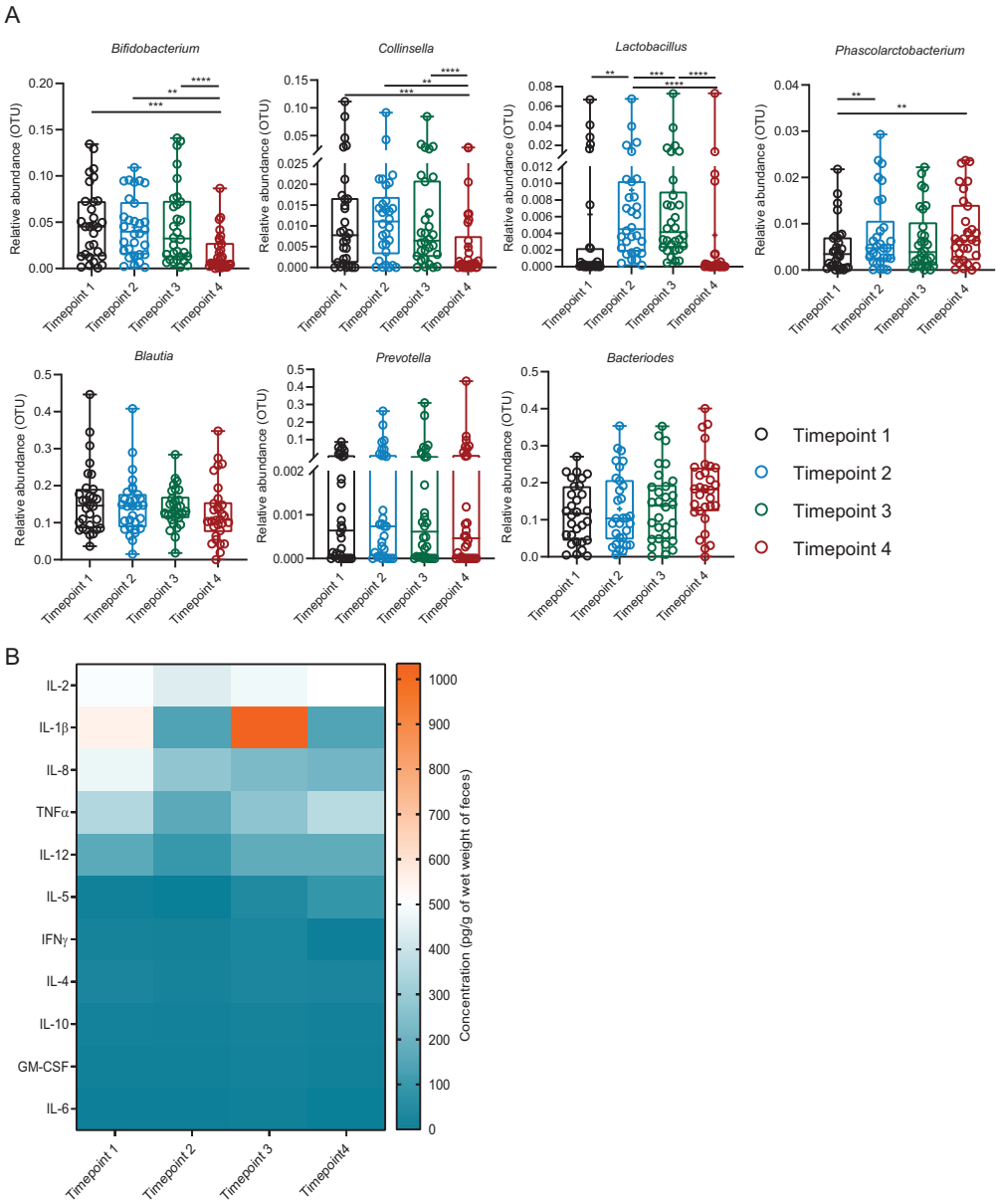

Figure 6. Profiles of faecal bacterial genera and cytokines of Cluster 3. (**A**) Comparison of relative abundance (OTUs) of seven major faecal bacterial genera of Cluster 3 across the time points. Different colours represent different time points. The bacteria which were significantly different between each time point (tested by Friedman rank-sum and post hoc Nemenyi multiple pairwise comparison tests) are presented as **** $p < 0.0001$, *** $p \geq 0.0001$–$p < 0.001$, ** $p \geq 0.001$–$p < 0.01$. (**B**) Comparison of concentration of faecal water cytokines (pg/g of wet weight of faeces) of Cluster 3 across the time points. Means of concentration are presented. No significant difference in the cytokine levels between the time points were found by the two-way analysis of variance (ANOVA) followed by Bonferroni multiple comparisons tests. IL = Interleukin, TNF = Tumour necrosis factor, IFN = Interferon, GM-CSF = Granulocyte-macrophage-stimulating factor. Timepoint 1 = baseline; 14 days after washout, Timepoint 2 = first 7 days after ingestion, Timepoint 3 = second 7 days after ingestion, Timepoint 4 = follow-up; 14 days after non-ingestion. $n = 30$ for each time point.

4. Discussion

The subjects recruited in the study were young adults mostly of the same race (Chinese: 61/75 = 81%), falling within a narrow range of age and basal metabolic rate (BMI). Moreover, subjects of different ethnicities (Malay and Indian) were found within the same microbiome clusters as those of the Chinese. Therefore, possible confounding factors, such as ethnicity, age and obesity, could be eliminated from the interpretation of the biodata and their association. It should be highlighted that *Prevotella* dominated Cluster 2 subjects were not heavier than *Bacteroides* Cluster 1, although BMI has been reported as negatively associated with *Bacteroides* [22]. The body weight of subjects was therefore not followed during the intervention study. Incidentally, most of the subjects provided stools of the healthy normal type (types 3 and 4, 55/75 = 73%), and abnormal stool types were randomly distributed among the three clusters, thus stool type was not a major factor for consideration in the clustering of microbiomes in this study.

Singapore is a multiethnic global city. It is not surprising that despite the Asian ethnicity, 41% of the young adults opted for a Western dietary habit, consuming more sugary beverages, meat, and carbohydrates, often in the form of Western fast foods, such as hamburgers, fried chicken, and potato. Their faecal microbiome is denoted as Cluster 1, dominated by *Bacteroides* (30% of total OTUs), having a microbiota profile like meat-eating Europeans and East Asians [11,12,23,24]. However, 19% of the young adults indulged in traditional Southeast Asian dietary habits, consuming more plant-rich foods, such as vegetables and fermented foods. Their faecal microbiome is denoted as Cluster 2, which was dominated by *Prevotella* (21% of total OTUs), typical of Southeast Asians [10,11]. The remaining belonged to Cluster 3 (40% of total subjects studied), who consumed an intermediary frequency of sugary beverages and vegetables in comparison with Clusters 1 and 2. The high consumption frequency of carbohydrate-rich and protein-rich foods is a reflection of regular consumption of both rice and bread (bun) or potato, and meat as burger and slices cooked with vegetables. Cluster 3 subjects did not possess a predominant bacterium but harboured a comparatively higher proportion of *Blautia* (16% vs. Cluster 1 and Cluster 2, 12% each), *Ruminococcaceae* (8% vs. Cluster 1, 3% and Cluster 2, 6%) and *Ruminococcus* (4% vs. Cluster 1 and Cluster 2, 1% each) and thus demonstrated a more evenly distributed microbiome profile. In earlier studies, subjects with a microbiome rich in mucin degrading *Ruminococcus* [12,18] were grouped under an enterotype purported to consume more alcohol and polyunsaturated fats.

Between the two predominant gut microbiota, *Prevotella* may have been diet determined, as it showed a strong correlation with most of the food types (carbohydrate, protein, vegetable, nuts and sugary beverages). The *Bacteroides* may be the secondary respondent, in response to the level of *Prevotella*.

The *Lactobacillus* consumed appeared to either reproduce or enhance the reproduction of endogenous *Lactobacillus* strains in the gastrointestinal tract of subjects in Cluster 1 and 3, as there was an increased abundance of *Lactobacillus* in the faecal samples during the consumption period in the *Lactobacillus* intervention study. The study was not able to verify the origin of this increase in *Lactobacillus* as strain-specific primers were not available. The abundance of *Lactobacillus,* however, could not be sustained and returned to that of the basal level within 14 days of stopping oral supplementation. This suggests that the *Lactobacillus* consumed could not colonise the gastrointestinal tract, a sign of colonisation resistance in the clusters.

The *Lactobacillus* was unable to propagate and colonise the gastrointestinal tract of Cluster 2 individuals, as the abundance of *Lactobacillus* in the faecal samples did not alter significantly throughout the *Lactobacillus* administration. This demonstrates that persistence and colonisation of the exogenous *Lactobacillus* in the gastrointestinal tract of Singapore young adults are basal microbiome and diet dependent. This agrees with our earlier studies [25,26].

The introduction of *Lactobacillus* was not able to alter the profile of major microbiota (>1% of total OTUs) in Cluster 1, demonstrating a stable microbiota structure. Consumption

of the *Lactobacillus* led to a minimal alteration in the level of cytokines. In short, the *Lactobacillus* showed minimal effects on Cluster 1 subjects in terms of microbiota composition and modulation in immune activities.

The introduction of the *Lactobacillus* to subjects in Cluster 2, however, reduced the abundance of *Prevotella* at Timepoint 4 and rendered the microbiota profile to resemble that of Cluster 3. *Lactobacillus* has been reported inhibitory to *Prevotella* [27,28]. The basal faecal water cytokine IL-2, -12 and TNFα levels in Cluster 2 were 3–6 times higher than the basal levels in Cluster 1. This may imply higher lymphocytic and pro-inflammatory activities in this cluster. This agrees with the proposal that gut *Bacteroides* exclude and protect the host from gastrointestinal pathogens [9], thus it may not be surprising that subjects in Cluster 2 depended on immunity for protection. The immunological activities were modulated upon consumption of the *Lactobacillus*, and the pro-inflammatory activities in the gastrointestinal tract seem to migrate from IL-8 neutrophil-mediated (down-regulated 3.8 times at Timepoint 4) to an IL-1β leukocytic mediated (up-regulated 7.2 times) pathway. The clinical relevance is, however, unclear.

Subjects in Cluster 3 consumed Western and Asian foods in rotation randomly, which is typical among Singaporeans [29]. Thus, a wide variation in the food component profile was observed among the subjects. A Singaporean may consume local Southeast Asian breakfast, English morning tea, American fast food lunch, a Japanese snack for afternoon tea, Chinese style dinner, Indian supper, and the sequence may alter the following day. This alternating dietary habit was not able to support a predominant microbiota, as in Clusters 1 and 2. The vacated ecological niches in the gastrointestinal tract were occupied by small but significant increases in the relative abundance of some major microbiota (>1% total OTUs), namely *Blautia, Ruminococcaceae* and *Ruminococcus*.

Gut microbiota diversity has been proposed as beneficial in maintaining the physiological functions and health of the human host [30,31]. Gut microbiota composition is largely determined by diet [10–13], however, it is difficult to achieve a sufficiently broad range of food types in one single meal to support microbiota diversity. Thus, it is expected that the human gut microbiome is mostly dominated by one microbiota, such as *Bacteroides* or *Prevotella*. The Singapore dietary habit of rotating food types represents an achievable and enjoyable approach in diversifying dietary components and even gut microbiota composition.

The Cluster 3 microbiome structure was not as stable as Cluster 1. Nevertheless, the introduction of the foreign bacterium led only to gradual reduction in the abundance of *Bifidobacterium* and *Collinsella*, but no change among the other major microbiota (>1% total OTUs), and it exhibited colonisation resistance to *Lactobacillus*, as the level of the exogenous *Lactobacillus* was reduced to that of the baseline after cessation of *Lactobacillus* consumption. It should be highlighted that both the alpha and beta diversities (weighted and unweighted) in Cluster 3 across the three time points (during consumption and after cessation of consumption of a large dosage of *Lactobacillus*) remained unchanged, which suggested that the microbiome in Cluster 3 was structurally as stable as that of Clusters 1 and 2. For immunity, except IL-1β at Timepoint 3, consumption of the *Lactobacillus* led to a general reduction of overall lymphocytic and pro-inflammatory activities.

From the perspective of lactic acid bacterium administration, the effect of the *Lactobacillus* on the gut microbiome was basal microbiome dependent. This agrees with our earlier study on the provision of a *Lactobacillus* to people across a wide distance of geographical area, from Mongolia to Singapore [25]. Moreover, the present study showed that immune modulation elicited by the *Lactobacillus* administration was also basal microbiome or immune status dependent. *Lactobacillus* appeared to have a larger immunoregulatory effect on people who belong to Clusters 2 and 3.

The dominating microbiota *Bacteroides* in Cluster 1 has been aligned with health benefits described in the Introduction; however, it is also listed as an independent high-risk factor for many common diseases in developed countries. These include arterial diseases [32,33], type-2 diabetes [34,35], colorectal cancer [36–38], cardiomyopathy [39],

rheumatoid arthritis [40], inflammatory bowel disease [41], Parkinson's disease [42], celiac disease [43] and Alzheimer disease [44]. On the other hand, Cluster 2 microbiome is dominated by *Prevotella*. Despite the fact that it positively interferes in energy homeostasis and glucose control [7,8], *Prevotella* is also implicated directly or indirectly in the causation of many chronic inflammatory diseases. These include periodontitis, bacterial vaginosis, rheumatoid arthritis, metabolic disorders [45], tonsillitis [46], advanced fibrosis non-alcoholic fatty liver disease (NAFLD) [47], cardiometabolic risk [48] and asthma [49]. This study thus brings about a critical question. Since the predominating bacterium, *Bacteroides* and *Prevotella* in Clusters 1 and 2 respectively, is associated with some forms of health benefit but also illness, and Cluster 3 microbiome demonstrated a more even distribution of major microbiota types and colonisation resistance, could Cluster 3 be a healthier microbiome structure? This warrants further study, as a means for the maintenance of healthy status. The relatively high economic standing and excellent medical care may explain Singaporeans being among the world's longest life expectancies at birth [50] (83.5 years according to the 2019 latest data). It could not be ruled out that a diverse microbiome without predominant disease-inducing microbiota contributes to Singaporeans having the status of the longest Healthy Life Expectancy (HALE) at birth [51] (76.2 years according to the 2016 latest data).

As demonstrated in this study, understanding the correlation between the gut microbiome, diet and health in multiethnic communities represents an excellent model system, due to the wider variety of food choices in a population of defined ethnicity, lifestyle and living environment. In summary, alternating dietary types over meals, as in Singaporean dietary habits, represents a practical approach in achieving a broad base dietary composition. This appeared to balance the gut microbiota profile, leading to a non-predominant microbiota type (enterotype).

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Institutional Review Board Statement: This study was conducted according to the guidelines of the declaration of Helsinki and approved by Institutional Review Board of National University of Singapore (NUS-IRB Ref. No. B-16-054; approved on 8 November 2015).

Informed Consent Statement: Informed consent was obtained from all participants involved in the study.

Data Availability Statement: All data relevant to this study were submitted as supplementary tables and the sequencing data were deposited at EBI repository (accession no: PRJEB39242).

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Abbreviations

GRAS	Generally regarded as safe
IL	Interleukin
TNFα	Tumour Necrosis Factor alpha
GM-CSF	Granulocyte-macrophage Colony-Stimulating Factor
IFNγ	Interferon gamma
BMI	Body Mass Index
OTU	Operational Taxonomic Unit
NAFLD	Non-Alcoholic Fatty Liver Disease
HALE	Healthy Life Expectancy
CFU	Colony Forming Unit
API	Analytical Profile Index
FFQ	Food Frequency Questionnaire
PBS	Phosphate-Buffered Saline
TE	Tris Ethylenediaminetetraacetic acid
HT1	Hybridisation Buffer
NaOH	Sodium hydroxide
PCR	Polymerase Chain Reaction
QIIME	Quantitative Insights Into Microbial Ecology
JSD	Jensen-Shannon distance
PAM	Partitioning Around medoids
CH	Calinski-Harabasz
Si	Silhouette coefficient
db-RDA	distance based Redundancy Analysis
PCoA	principal coordinate analysis
PMSF	Phenyl-methyl-sulfonyl fluoride
BSA	Bovine Serum Albumin
LLOQ	lower limit of quantification
PERMANOVA	Permutational Multivariate Analysis of Variance
ANOVA	Analysis of Variance

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A single serving of mixed spices alters gut microflora composition: a dose-response randomised trial.
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A single serving of mixed spices alters gut microflora composition: a dose–response randomised trial

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Short-term changes in dietary intake can induce changes in gut microbiome. While various dietary polyphenols have been shown to modulate gut microflora, the acute influence of polyphenol-rich mixed spices has not been explored in a controlled setting. We investigated the effects of a single serving of mixed spices Indian curry consumption, in two separate doses, on the gut microbiome in 15 healthy, Singaporean Chinese males, with age and BMI of 23.5 ± 2.4 years and 22.9 ± 2.2 kg/m^2 respectively. We found that a low-polyphenol, no spices Dose 0 Control (D0C) meal led to an increase in *Bacteroides* and a decrease in *Bifidobacterium*. In comparison to D0C, there was significant suppression of *Bacteroides* ($p < 0.05$) and an increase in *Bifidobacterium* ($p < 0.05$) with increasing doses of curry meal Dose 1 Curry (D1C) and Dose 2 Curry (D2C) containing 6 g and 12 g mixed spices respectively. Significant correlations were also found between bacterial changes and plasma phenolic acids. No differences between treatments were observed in the alpha-diversity of the gut microflora. This study has shown that a single serving of mixed spices can significantly modify/restore certain commensal microbes, particularly in people who do not regularly consume these spices.

The gut microbiome has been widely acknowledged as a dynamic and complex community of bacteria that is dependent on extrinsic and intrinsic factors, of which diet is one of the most dominant modulators[1–3]. Several previous studies have shown that dietary patterns can affect gut microbiome[4–7]. Numerous studies indicate fruits[8–10] and/or vegetables[11–14] as well as whole grain cereals[12,15] can directly affect gut microflora composition. Besides these dietary patterns, foods and established nutrients, various 'non-nutrient bioactive compounds' in diet, including polyphenols, can significantly modulate gut microflora composition[16–18]. Spices that are often used as culinary ingredients for flavouring, colouring or prolonging food shelf-life[19] are rich in polyphenols that are generally poorly absorbed by the upper intestine and present in relatively high concentrations in the lower gut[20]. Therefore, they are expected to interact and modulate the gut microbiome.

Depending on the polyphenol type, they may either have antimicrobial effects towards certain gut bacteria, whereas, stimulatory effects on other types of bacteria[21,22], as well as having the ability to alter the ratios of *Firmicutes*/*Bacteroidetes*[23] and *Prevotella*/*Bacteroides*[24]. These studies highlighted the potential modulatory role of polyphenol-rich spices on the composition of the human gut microbiome. Given that nowadays, spices are widely consumed globally, albeit on an occasional basis, it is therefore important to investigate the acute effects of spice consumption on the gut microbiome. Most previous research examined the effect of single spices or polyphenolic extracts from single spices such as curcumin on both human subjects and in animal studies[18,21,25,26]. However, few prior studies have examined the effects of introducing a dietary concoction of several spices in combination, which is how they are typically consumed in different curries across various cultures worldwide. To the best of our knowledge, only one recent study has attempted to test the effects of five different mixed spices in combination (cinnamon, oregano, ginger, rosemary, black pepper and cayenne pepper), in dietary doses, on gut microbiome and found that up to 26 operational taxonomic units (OTUs) were modulated as a result of the mixed-spice treatment compared with placebo[27].

Therefore, we investigated the acute effects on gut microflora of a combination of 7 different spices as a secondary analysis of a recently completed study that measured various metabolic outcomes[28,29]. Considering that

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Measurement	Mean ± SD			p value (one-way ANOVA; mixed-effects analysis)
	Dose 0 control (D0C) (n = 14)	Dose 1 curry (D1C) (n = 15)	Dose 2 curry (D2C) (n = 15)	
Energy (kcal)	1763.600 ± 377.666	1572.576 ± 429.014	1560.602 ± 406.569	0.1384
Carbohydrates (g)	189.385 ± 38.211	179.072 ± 51.176	183.486 ± 78.424	0.8262
Protein (g)	88.338 ± 27.345	78.561 ± 19.003	76.277 ± 20.264	0.1771
Total fats (g)	67.668 ± 19.673	57.709 ± 20.035	59.500 ± 20.667	0.2109
Saturated fats (g)	27.000 ± 9.586	23.832 ± 8.562	24.982 ± 7.659	0.5338
Fibre (g)	15.726 ± 6.151	13.969 ± 6.380	15.566 ± 7.358	0.417
Cholesterol (mg)	364.247 ± 165.379	301.156 ± 119.466	333.020 ± 162.609	0.2956
Sodium (mg)	3191.363 ± 766.049	2910.272 ± 861.957	3163.564 ± 1007.169	0.5686

Table 1. Mean daily intake of energy and nutrients during the run-in periods prior to each intervention session. P values were calculated from the statistical test of one-way ANOVA based on the consumption of each nutrient. D0C = Meal with no spices intervention, D1C = Meal with low (6 g) mixed spices intervention, D2C = Meal with high (12 g) mixed spices intervention, n = 15 each intervention, except for D0C, where the data from 1 subject was excluded because of being an outlier (extreme over-reporting).

this study was undertaken in an acute setting with two separate mixed spices doses, it provided an ideal setting to investigate acute, dose-dependent changes in gut microbiome as a result of the consumption of these mixed spices. From this same study, since we have previously reported dose-dependent increases in two phenolic acids (i.e., cinnamic acid (CNA) and phenylacetic acid (PAA)) as well as increase in the total polyphenol excretion (TPE)[29,30], we have also investigated the associations between these parameters as objective markers of the spices intake with the changes in the gut microbiome.

Results

Basic demographics and dietary intake 3-days prior to each session. The mean age and BMI of the participants at the first session who provided the stool samples were 23.5 ± 2.4 years and 22.9 ± 2.2 kg/m^2 respectively. The reported intakes of energy and some key nutrients during the 3-day 'run-in periods' immediately prior to the main study day (Day 1) are shown in Table 1. There were no significant differences between the intervention sessions in any of the nutrients or energy intake.

Changes in microbiome composition. There were no significant differences in the relative abundances of the individual bacterial genera at baseline (Day 0) or at Day 2 as calculated using Friedman's test with Dunn's multiple comparison as shown in Fig. 1 and Supplementary Tables S1 and S2. The alpha diversity of each spices intervention is shown in Fig. 2. No significant differences were found between the various intervention sessions for both the Chao 1 and Shannon diversity indices.

However, the spice intervention led to significant changes in the relative abundances of two bacterial genera. The changes from Day 0 to Day 2 in the relative abundance of the various bacterial genus (Operational Taxonomic Units: OTUs) during each of the three intervention sessions are shown as a heatmap in Fig. 3.

As can be seen in the heatmap (Fig. 3), compared with the control, the relative abundance of *Bacteroides* was significantly reduced with increasing spice doses (adjusted $p < 0.05$ for both D0C vs D1C and D0C vs D2C). This was mainly driven by the fact that there was an increase in *Bacteroides* relative abundance changes from Day 0 to Day 2 during the D0C session, whereas the *Bacteroides* relative abundance from Day 0 to Day 2 either remained the same or decreased slightly during D1C and D2C sessions. On the contrary, the relative abundances of *Bifidobacterium* (adjusted $p < 0.05$ for D0C vs D2C) increased significantly with spice intake as compared with control. This was also driven by the fact that there was a decrease in *Bifidobacterium* relative abundance changes from Day 0 to Day 2 during the D0C session, whereas the *Bifidobacterium* relative abundance from Day 0 to Day 2 either remained the same or increased slightly during D1C and D2C sessions. The extent and the variabilities in the relative abundances in these two genera during the various intervention sessions at Day 0 and 2 are shown in Fig. 4 as median and inter-quartile range (IQR). The paired individual changes in relative abundances between two time points (Day 0 to Day 2) for three spices doses in these two genera is shown in Fig. 5.

Figure 6 shows the changes in relative abundances of (a) *Bacteroides* and (b) *Bifidobacterium* in relation to their absolute relative abundance at baseline (Day 0) for all three doses. We found a significant correlation ($R^2 = 0.284$, $p = 0.041$) for D2C in *Bacteroides* but, no such correlation for D0C. On the other hand, those with low *Bifidobacterium* relative abundance at baseline had the greatest changes in relative abundances for D0C ($R^2 = 0.577$, $p = 0.001$) and D2C ($R^2 = 0.433$, $p = 0.008$).

Association between changes of bacterial genus and two plasma phenolic acids and changes urinary total polyphenol excretion in 24 h. As shown in Table 2, when we pooled the data across all intervention time points, we found significant positive associations between plasma concentration (on Day 1) of CNA and *Bifidobacterium* whereas negative associations between plasma CNA and *Bacteroides*, *Ruminococcus* and *Oscillospira*. Similarly, plasma PAA concentration was also negatively associated with *Bacteroides, Lachno-*

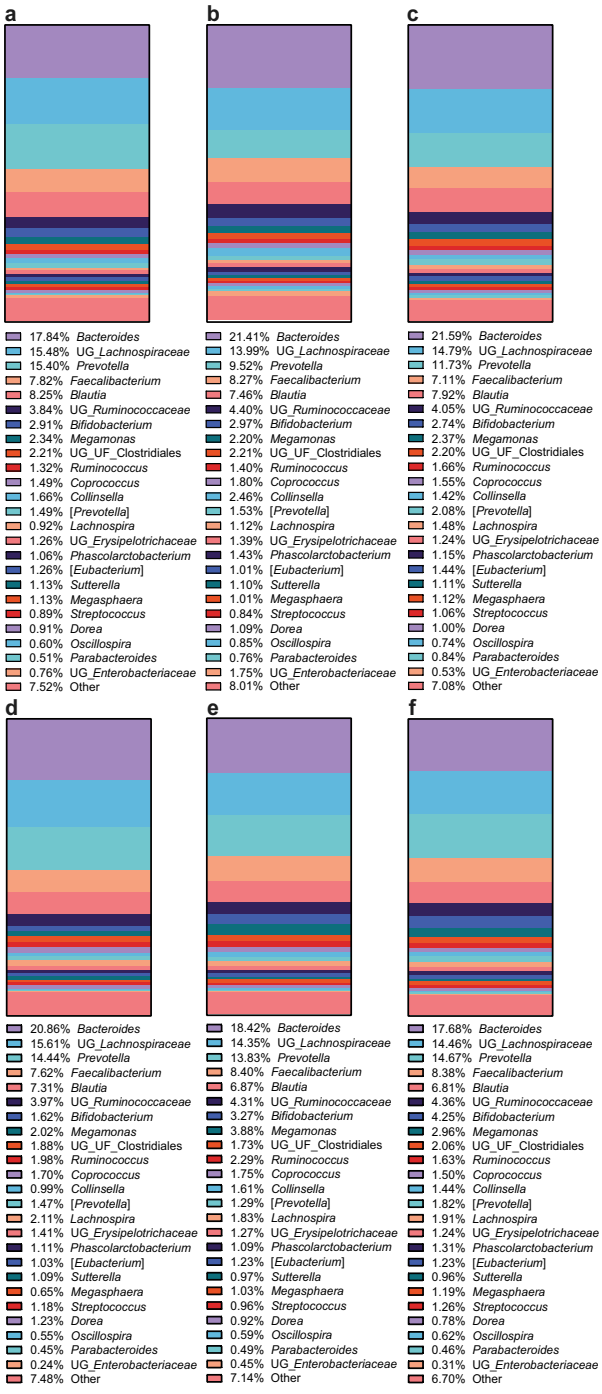

Figure 1. Comparison of the relative abundance of bacterial genera for the three different doses of spices intervention at Day 0 (baseline) (a-c) and Day 2 (d-f) for D0C, D1C and D2C respectively. 'Other' was categorised as less than 1% of bacterial OTUs among the population. Bacteria at baseline or at Day 2 were not significant different between intervention sessions. D0C = Meal with no spices intervention, D1C = Meal with low (6 g) mixed spices intervention, D2C = Meal with high (12 g) mixed spices intervention, *UG* unknown genus, *UF* unknown family. n = 15 each intervention.

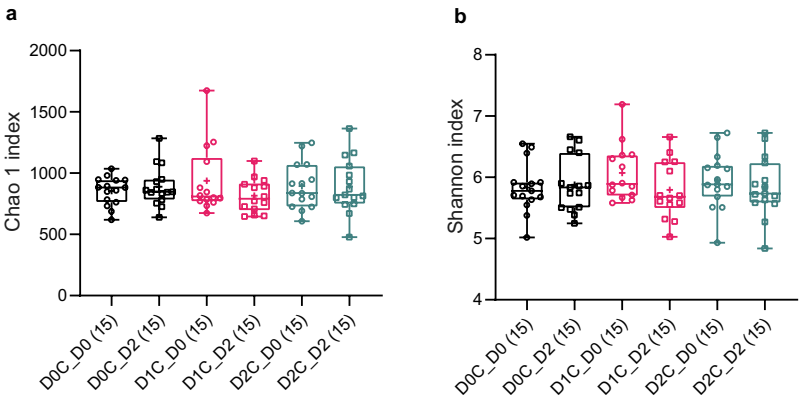

Figure 2. Alpha diversity of the three different doses of spices intervention (**a**) Chao 1's and (**b**) Shannon's indexes were measured and described at Y axis. No significant difference between spices intervention were found by Friedman's test with Dunn's multiple comparison within Day 0 or Day 2. The types and days of intervention were drawn as different symbols and colours. + shows mean. D0C = Meal with no spices intervention, D1C = Meal with low (6 g) mixed spices intervention, D2C = Meal with high (12 g) mixed spices intervention. No of samples were in the parenthesis.

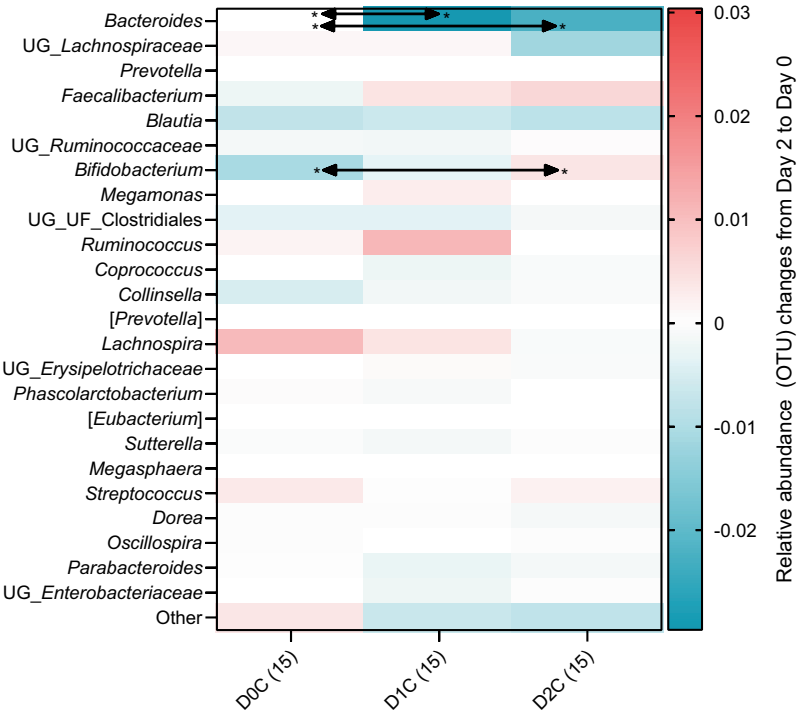

Figure 3. Heatmap of medians relative abundance changes of the major bacterial genera after two days of intervention for the three doses of mixed spices intervention. 'Other' was categorised as less than 1% of bacterial OTUs among the population. The relative abundance changes (Day 2 minus Day 0) in bacteria between various doses were analysed using Friedman's test with Dunn's multiple comparison and described as * adjusted $p < 0.05$.

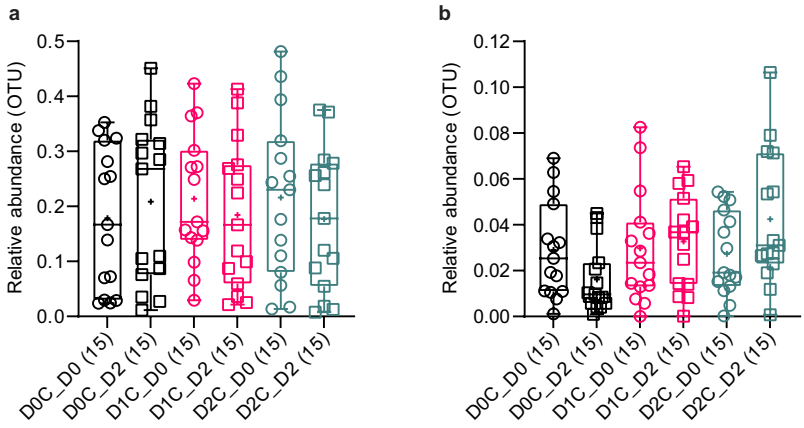

Figure 4. Relative abundance distribution of (**a**) *Bacteroides* and (**b**) *Bifidobacterium* at Day 0 and Day 2 for three doses of mixed spices intervention. No bacteria were significantly different between three different doses of spices intervention analysed by Friedman's test with Dunn's multiple comparison. D0C = Meal with no spices intervention, D1C = Meal with low (6 g) mixed spices intervention, D2C = Meal with high (12 g) mixed spices intervention. *UG* unknown genus, *UF* unknown family. No of samples (each intervention) was 15 and described in the parenthesis.

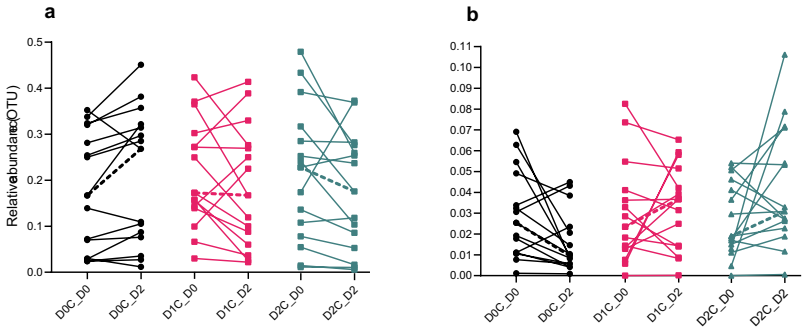

Figure 5. Paired individual changes in relative abundance between two time points (Day 0 to Day 2) for three spices doses (**a**) *Bacteroides* and (**b**) *Bifidobacterium*. Wilcoxon matched-pairs signed rank test was applied and significant pairs were described as * $p < 0.05$. D0C = Meal with no spices intervention, D1C = Meal with low (6 g) mixed spices intervention, D2C = Meal with high (12 g) mixed spices intervention. n = 15 each intervention. Dashed line indicates median change.

spira and *Phascolarctobacterium*. On the contrary, only *Coprococcus*, had a significant negative association with changes in 24 h urinary total polyphenol excretion (Day 1–Day 0).

Discussion

While polyphenolic compounds in diet are known to modulate gut microbiome[16–18], metabolism of dietary polyphenols also rely on the gut microbiome to exert their biological effects in humans, particularly for their breakdown into secondary metabolites and subsequent absorption[31]. This bi-directional association between dietary polyphenols and the gut microbiome is integral to the host-gut bacteria symbiosis. The present study is one of the few studies in humans investigating the influence of polyphenol-rich mixed spices, contained within curry meals, in two separate doses on acute changes in gut microflora. To the best of our knowledge, there has been only one other similar previous study undertaken with mixed spices in humans[27]. While this previous study by Lu et al. explored the effects of mixed spices consumption (5 g/day) over a longer time frame (2 weeks), our study showed that even a single meal containing mixed spices can modify/restore gut microflora within a period of 24–48 h. Moreover, our study had a rigorous study design of avoiding polyphenol-rich foods 3-days prior to each intervention session (i.e., during run-in period) and the rest of the meals during the measurement days apart from the intervention (D0C, D1C, and D2C) test meals were standardized and provided. Given that our

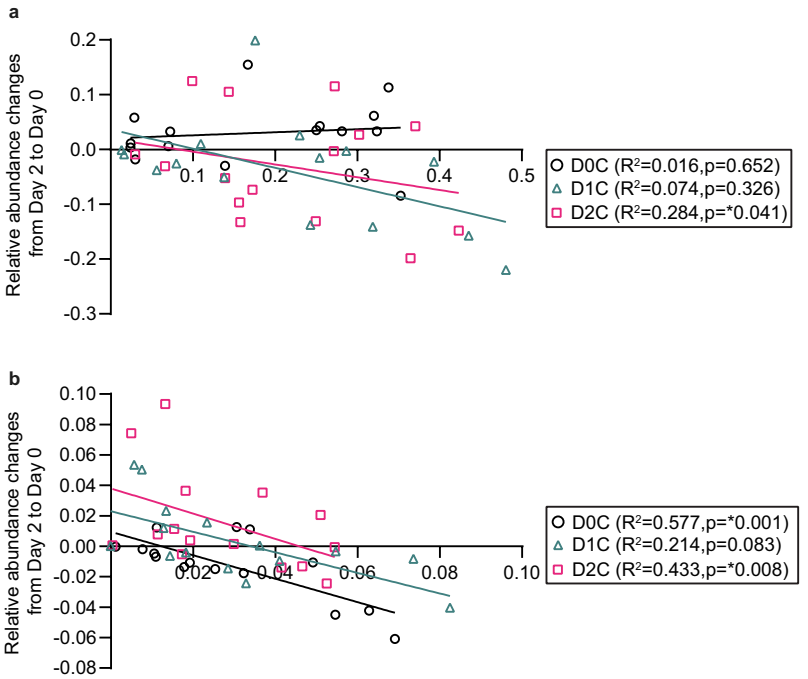

Figure 6. Changes in relative abundances of (**a**) Bacteroides and (**b**) Bifidobacterium in relation to baseline relative abundance for the three doses. Simple linear regression was applied and R^2 and p values were described for the group that had $*p < 0.05$. The relative abundance of bacteria at Day 0 and relative abundance changes were represented at X and Y axes, respectively. The colours represent the respective spices intervention session. D0C = Meal with no spices intervention, D1C = Meal with low (6 g) mixed spices intervention, D2C = Meal with high (12 g) mixed spices intervention. No of samples (each intervention) was 15.

study was undertaken in a Chinese population who otherwise consumed Indian mixed spices less frequently, it minimised any residual confounding due to prior habituation of mixed spices intake. It appears from our results that a continuous adherence of a low polyphenol diet during the D0C (control) session led to an increase in the relative abundance of *Bacteroides* and a decrease in the relative abundance of *Bifidobacterium* between Days 0 to 2 of the intervention, whereas the re-introduction of polyphenol rich spices at the two doses of curry somewhat restored/reversed this trend within this same time frame. The rapid time frame of this change in gut microbiome as a result of a single bout of curry, at 2 separate doses, is not surprising given that previous studies have also shown alterations in gut microflora within a 24-h time scale[32–34].

Prior to their human trial, Lu et al. undertook a separate study in vitro which showed prebiotic potential, altering greater than 120 bacterial species, including the growth promotion of certain probiotics such as *Bifidobacterium* spp. and inhibition of growth of pathogenic bacteria such as *Clostridium* spp.[35]. In their human trial, the same authors found that the mixed spices affected 26 OTUs, including growth promotions of *Bifidobacteria* spp., *Lactobacilli* spp., *Bacteroides* spp. and inhibition of *Clostridia* spp.[27]. In our trial, while *Bifidobacterium* also increased in the highest dose of mixed spices (D2C) vs control (D0C), there were significant reductions in *Bacteroides* (in both D0C and D2C vs D0C) populations with increasing mixed spices dose. The variations in the findings between the two studies may be due to differences in the background diet, the initial bacterial populations and/or differences in host genetics. *Prevotella* and *Bacteroides* are the two main genera of the same *Bacteroidetes* phylum and are highly abundant in human stool samples[36]. It is generally accepted that vegetable-rich diets tend to give rise to a greater abundance of *Prevotella* whereas animal-based diets tend to be enriched in *Bacteroides*[37,38]. Similarly, vegetarians and vegans have greater *Prevotella/Bacteroides* ratio than Omnivore populations[39,40], as well as rural populations having a greater *Prevotella/Bacteroides* ratio as compared with urban populations[34,41]. However, we did not find any differences in *Prevotella/Bacteroides* ratio in our study most likely due to dietary fibre in the above-mentioned dietary patterns driving such effects.

Indeed, previous studies found that increasing the intake of various polyphenols, such as dealcoholized red wine extracts rich in resveratrol[42] and pomegranate rich extracts[43] led to increases in *Prevotella* populations, although we did not observe this effect within our study population. In support of our study, a previous study reported that in vitro incubation of dietary polyphenolic components such as chlorogenic acid, caffeic acid,

	Changes in urinary total polyphenol excretion (Day 1 minus Day 0)		Plasma cinnamic acid $AUC_{0-7\,h\ Day\ 1}$		Plasma phenyl acetic acid AUC $_{0-7\,h\ Day\ 1}$	
	Spearman r	p values (two-tailed)	Spearman r	p values (two-tailed)	Spearman r	p values (two-tailed)
Bacteroides	− 0.173	0.224	− 0.300	*0.038	− 0.299	*0.033
UG_*Lachnospiraceae*	0.104	0.467	− 0.097	0.512	− 0.052	0.717
Prevotella	0.186	0.192	0.075	0.613	0.015	0.914
Faecalibacterium	0.098	0.493	− 0.189	0.199	0.133	0.354
Blautia	− 0.260	0.065	0.013	0.929	− 0.055	0.703
UG_*Ruminococcaceae*	− 0.050	0.725	− 0.019	0.900	0.187	0.189
Bifidobacterium	0.076	0.598	0.372	**0.009	0.222	0.117
Megamonas	0.137	0.339	0.183	0.214	− 0.053	0.714
UG_UF_*Clostridiales*	− 0.220	0.121	0.179	0.224	− 0.063	0.661
Ruminococcus	− 0.007	0.961	− 0.308	*0.033	− 0.016	0.909
Coprococcus	− 0.298	*0.034	0.122	0.411	0.153	0.283
Collinsella	− 0.033	0.818	0.269	0.065	0.255	0.071
[Prevotella]	0.065	0.649	− 0.254	0.082	0.181	0.205
Lachnospira	0.167	0.243	− 0.255	0.081	− 0.284	*0.043
UG_*Erysipelotrichaceae*	− 0.128	0.370	− 0.087	0.556	− 0.194	0.173
Phascolarctobacterium	− 0.205	0.149	− 0.136	0.355	− 0.354	*0.011
[Eubacterium]	− 0.231	0.102	− 0.146	0.322	0.062	0.667
Sutterella	− 0.032	0.825	0.272	0.061	− 0.022	0.879
Megasphaera	0.133	0.353	0.226	0.122	0.192	0.177
Streptococcus	− 0.140	0.329	0.112	0.449	− 0.194	0.173
Dorea	− 0.255	0.071	− 0.239	0.101	− 0.068	0.635
Oscillospira	− 0.062	0.664	− 0.288	*0.047	0.112	0.435
Parabacteroides	− 0.068	0.637	− 0.153	0.298	0.044	0.761
UG_*Enterobacteriaceae*	− 0.004	0.978	0.029	0.843	0.096	0.504
Other (< 1% of total bacteria)	− 0.165	0.248	− 0.141	0.340	0.089	0.533

Table 2. The correlation between changes in 24 h urinary total polyphenol excretion, plasma cinnamic acid $AUC_{0-7\,h,}$ and plasma phenyl acetic acid $AUC_{0-7\,h}$ and relative abundance changes of the bacterial genus from Day 0 to Day 2 of mixed spices intervention, for all intervention doses combined. Non-parametric Spearman correlation coefficient (r) and two-tailed p values were shown. *AUC* area under curve, *UG* unknown genus, *UF* unknown family. n = 15 each intervention. **p ≥ 0.001– < 0.01, *p < 0.05.

and rutin reduced the *Bacteroides* population under anaerobic conditions[44]. In vivo studies in rats also found a decrease in *Bacteroides* population upon feeding of polyphenol-rich cocoa[45]. Even within our lab, we have shown that tea phenolic metabolites can extensively suppress the growth of *Bacteroides*[22]. Thus, taking into account these previous findings, the modulation of *Bacteroides* by polyphenol-rich mixed spices observed in our present study is likely to be causally associated and further studies are needed to confirm this. It should also be noted that the median reduction in relative abundance of *Bacteroides* was driven by a subset of voulnteers (Fig. 5a) who mainly had higher level of this bacteria at Day 0 as discussed in detail below and presented in Fig. 6a. This is further supported by no differences in the median *Bacteroides* relative abundance between various spice dose at Day 2 (nor Day 0 as expected, as shown in Fig. 5a). The negative associations between both CNA and PAA with *Bacteroides* further reiterates the decreases in the relative abundance of this bacteria with increasing doses of mixed spice rich curries may well be causally linked, as we have previously shown that the concentrations of both CNA and PAA could be used as objective biomarkers of the intake of the mixed spices used in our study[30].

Several previous studies have indicated that polyphenol-rich foods can increase the abundance of *Bifidobacterium*[46]. Among the randomized controlled trials, one study with dealcoholized red wine showed significant increases in *Bifidobacterium*[47], which was in fact further associated with increases in secondary metabolites of anthocyanins including several phenolic acids[48]. Similarly, consumption of wild blueberries[49] as well as tart cherries[50] were also shown to increase *Bifidobacteria*. Furthermore, the study by Lu et al.[27] with five mixed spices, mentioned earlier, also found increases in the abundance of several *Bifidobacteria spp*. This, together with the findings of our study of the increases in *Bifidobacteria* with the highest dose of spice (D2C) meal indicates that polyphenol-rich spices may have bifidogenic properties. Hence, our study supports the concept of polyphenols having prebiotic properties, as discussed previously[51]. However, it should also be noted that the median increase in relative abundance of *Bifidobacterium* at higher dose of spices was mainly driven by only a subset of voulnteers, with low relative abundance at baseline (as shown in Fig. 5b), as discussed in more detail below (and shown in Fig. 6b). This is further supported by no differences in the median *Bifidobacterium* relative abundance between various spice dose at Day 2 (nor Day 0 as expected, as shown in Fig. 5b). Nonetheless, the positive association

between plasma CNA and *Bifidobacteria* could also be a causal effect, since various hydroxycinnamic acids such as chlorogenic acid and caffeic acid have been previously shown to stimulate the growth of *Bifidobacteria*[44].

In our study, the consumption of curry meals did not alter the alpha diversity of the gut microbiome of the tested subjects (Fig. 1). This may be due to the acute nature of the intervention as well as human gut microbiome, particularly the core members which account for most of the abundance in the gut microbiome that has been found to possess a high functional response diversity through functionally genetic redundancy[52]. This allows them to adapt well to short-term changes in the diet, which could explain why there were rarely large-scale changes in gut microbiota profile despite daily and seasonal variation in dietary ingredients and preparation[53,54]. Despite this adaptive capability, we have shown that a single bout of mixed spices can lead to, or restore compared to control dose (D0C session) significant modulation of certain gut microbes, particularly at the higher dose of curry (i.e., D2C meal). However, it remains to be established whether similar changes occur following longer-term consumption of polyphenol-rich spices and whether such changes are stable over time. Indeed this is one of the main limitations of this study in that finding reported here only describes acute modifications in gut microbiome. Another limitation of this study is that we only looked at associations of gut microbial changes with specific targeted metabolites in plasma/urine. Therefore future studies with individual and/or mixed spices should not only be undertaken with a longer duration of dietary intervention but should also explore how the changes in gut microbiome may be resulting from global changes in the metabolome in plasma and/or stool samples, arising from the intake of specific foods and/or food groups, in order to determine known/unknown metabolites that may directly influence certain bacterial populations. Nonetheless, with the globalization of dietary habits and the increasing amount of spices being consumed globally, the data from our study indicate an opportunity for bringing about beneficial changes in the gut microbiome through the intake of polyphenol-rich spices. Moreover, characterization of the gut microbiome across various parts of the world and various ethnic groups and/or cultures is an expanding area of research[43,55] and further work needs to be done on the extent to which specific diet and lifestyle practices can modulate microbiome within these populations. Importantly, the functional consequences of the modulation of the microbiota, including what constitutes a 'healthy microbiome' are yet to be fully established[56,57]. Furthermore, recent reviews highlighted the utility of these polyphenol-associated gut microbiota changes on several anthropometric and clinical parameters related to cardiometabolic health[18,58,59] and therefore changes in gut microbiome may be one of the major mechanisms through which dietary polyphenols may exert their widely reported benefits. The findings in our study also support these previous reports that some downstream metabolites of dietary polyphenols present in the mixed spices such as CNA and PAA may be directly influencing the bacterial changes observed with the intake of mixed spices used in our study.

Finally, the relative abundance at baseline of both *Bacteroides* and *Bifidobacterium* influenced the extent to which the changes in relative abundances of these two bacteria occurred at the various doses of curry. The regression plot in Fig. 6a shows a greater decrease in the relative abundance in *Bacteroides* in those individuals with a greater baseline relative abundance in this bacteria, particularly for Dose 2 curry, although this association was not present during the Dose 0 control session. Similarly, the regression plot in Fig. 6b shows that those with a lower relative abundance of *Bifidobacterium* had the greatest increase in the relative abundance (positive change) of the bacteria between Day 0 and Day 2 for both Dose 1 curry and Dose 2 curry. In comparison, majority of the individuals during Dose 0 control either had a reduction (negative change) in the *Bifidobacterium* relative abundance or did not change. This further highlights that the effects of dietary components on gut bacteria are likely to be dependent on the individual microbiome profile and hence may explain the inter-individual differences in responses to various dietary interventions.

Conclusions

This dose–response study has shown that even a single dose of mixed spices curry can modify/restore gut microbiome as compared with a control diet which was low in polyphyenols, within a 24–48-h timeframe. While the overall alpha-diversity of gut microbiome did not change as a result of this dietary intervention, there were in fact decrease/restoration in *Bacteroides* populations with increasing mixed spices doses relative to the dose 0 control (D0C) diet, when there was an increase in this bacteria. Similarly, our study also showed an increase/restoration in *Bifidobacteria* populations in the highest curry dose (D2C) compared with control (D0C), which further supports the bifidogenic potential of polyphenols. It should also be noted that the relative abundance changes in both *Bacteroides* and *Bifidobacterium* observed within each individual dose of spices (D0C, D1C and D2C) were mainly driven by a subset of individuals (Figs. 4 and 5), indicating large intra and inter-individual variabilities in responses to the dietary intervention. The reasons for this variability remains to be additionally investigated and was beyond the remit of this study. Furthermore, longer term studies are required to confirm our acute findings and a more detailed array of metabolites should be measured in order to establish mechanisms responsible for such changes.

Methods

Dietary intervention design. The study protocol, including the inclusion and exclusion criteria, has been detailed elsewhere[15]. In brief, the study was a randomized, crossover, acute, food based intervention trial specifically undertaken in 21–40-year-old, healthy, Chinese men, with a BMI between 18.5 and 27.5 kg/m^2, who would otherwise typically not consume large amounts of Indian spices as part of their habitual diet. This secondary analysis was only undertaken in a subset of 15 volunteers who provided stool samples for the study and have completed all three doses of the dietary intervention. A per-protocol approach was utilized and data from three volunteers were excluded. These volunteers did not complete all 3 doses of intervention since we used matching analyses across all 3 doses. The study was approved by the Domain Specific Research Board (DSRB) ethics committee, Singapore (Reference: C/2015/00729) and was registered at clinicaltrials.gov (ID: NCT02599272)

and was undertaken in accordance with the Declaration of Helsinki, revised in 2013 and as per Singapore Good Clinical Practice Guidelines. Informed consents from all volunteers were obtained before the intervention study.

Each volunteer in the study undertook 3 intervention sessions which were completed in random order which was obtained using an online randomization generator (http://randomizer.org). These sessions included a Dose 0 Control (D0C, no spices) session, Dose 1 Curry (D1C, low spices) session, and the Dose 2 Curry (D2C, high spices) session. The test meals for the D1C and D2C sessions included a mixture of 7 dried spice powders at doses 6 g and 12 g respectively. The spice mix was made up of turmeric, cumin, coriander, *amla* (Indian gooseberry), cinnamon, clove, and cayenne pepper mixed in the ratios of 8:4:4:4:2:1:1 respectively. The individual polyphenols present in the ingredients used to prepare the various curries (D1C and D2C) or the control (D0C) are listed in Supplementary Table S3.

The schematics of the study design is shown in Supplementary Fig. S1. In the 3 days prior to the main study day (Day 1) when the mixed spice containing meals (D1C or D2C) or the control meal (D0C) were consumed, all participants were asked to avoid consumption of any spice or any other polyphenol-rich foods (i.e., the 'run-in period'). To aid their compliance, a list of common foods rich in polyphenols and common spices were provided to them. Furthermore, a 3-day food diary was completed during the 'run-in period' for each study session to record all foods that they consumed to further facilitate the detection of erroneous spices or polyphenol-rich food consumption during these periods and to improve compliance to the dietary intervention. One day before the main study day of each session, the volunteers were required to provide their baseline (Day 0) stool sample prior to the food based intervention on Day 1. On the main study day (Day 1), in the morning after an overnight fast, the volunteer consumed one of 3 intervention meals (D0C, D1C or D2C test meals). The total energy, macronutrients as well as total vegetable contents of the 3 intervention meals were comparable as reported previously[15]. The rest of the meals and snacks on Day 1 were standardized and contained low amounts of polyphenol containing ingredients and no spices within them. On the following day (Day 2), another stool sample was provided. Therefore, Day 0 stool represented the gut microbiome of the participants prior to each intervention session, after abstaining from polyphenol-rich foods and spices for the previous three days, whereas, Day 2 stool sample represented the gut microbiome immediately after each dietary intervention. Between each study session, each participant had at least a 14-day 'washout period' during which the participants resumed their habitual diet. This was done to avoid any 'carry-over' effect from one intervention session to the next.

Polyphenol analyses in urine and plasma. Urine samples were also collected over 2 × 24 h periods between Day 0 to Day 2, in 2 × 3 L plastic containers (Simport, Canada), to measure total polyphenol excretion (TPE) using the Folin-Ciocalteu assay as described in detail previously[29]. 'Day 0' urine was collected in the first urine container from the morning of Day 0 morning until Day 1 morning immediately prior to the consumption of test meals. 'Day 1' urine was collected during the subsequent 24 h period immediately after the test meal consumption between Day 1 morning until Day 2 morning. Blood samples were also collected in K_2 EDTA vacutainer tubes (BD, Franklin Lakes, NJ, USA) immediately after the consumption of test meals on Day 1, at regular intervals (12 time points) to measure concentrations of various phenolic acids in plasma using a UHPLC-MS/MS method, as described in detail previously[30]. The areas under the curve (AUC) of postprandial concentrations of plasma phenolic acids were calculated using the trapezoid method.

Stool sample collection and 16 s rRNA gene sequencing analysis. For each stool sample collection (Day 0 and Day 2), the volunteers were asked to provide approximately 5 g of stool sample which was dissolved in a universal tube containing 2 ml of RNAlater solution (Ambion, Inc., Texas, USA) and were analysed for gut microbiota profiling with 16 s rRNA gene sequencing. To do this, 0.2 ml of faecal homogenate from approximately 1 g of collected stool was extracted using the phenol–chloroform method after washing with Phosphate-buffered saline (PBS). Along with the glass-beads mechanical extraction, Tris-SDS, TE-saturated phenol (Sigma-Aldrich, Cor., St.Louis, Missouri, USA) and phenol/chloroform/isoamyl alcohol (25:24:1) solutions were used. DNA was precipitated with sodium acetate and isopropanol followed by washing with ethanol and eluted in TE. The quantified DNA was normalised to 12.5 ng and amplified with a primer set that targeted at the regions of v3 and v4 of the 16 s rRNA gene. Once the amplicons were purified with Agencourt AMPure XP beads (Beckman Coulter, Inc., Fullerton, CA, USA), they were amplified with the Nextera XT index primers (Illumina, Inc., San Diego, USA). The quantified and normalised library was denatured and spiked with the PhiX control library followed by sequencing in the Miseq system (Illumina, Inc., San Diego, USA).

Bioinformatics and statistical analyses. The 16 s rRNA gene sequence outputs were analysed with Quantitative Insights Into Microbial Ecology (QIIME) version 1.9.1[60]. After joining the reads and quality filtering, the chimeric sequences were removed with USEARCH v6.1[61]. The non-chimeric sequences were picked to get the operational taxonomic unit (OTU) using 97% similarity sequences of Greengenes v13_8 database. The assigned OTUs were summarised into the taxonomical category for the bacterial profiling of samples in three intervention sessions. Chao 1 and Shannon diversity indexes of alpha diversity were computed using the OTU table. The 3-day food diary data were converted into the average energy and nutrients values per day by an online tool of energy and nutrient composition of food Health Promotion Board, Singapore[62].

The bacterial genus taxonomy data, alpha diversity data and the dietary nutrients data were further, analysed and normality tests were performed. Bacterial genus with more than 1% of the relative abundance of the total bacterial population were included in this study and 24 major bacterial genera were found. Mean and Standard Deviation (SD) were described for the average energy and nutrients such as energy (kcal), protein (g), total fats (g), saturated fat (g), fibre (g), carbohydrate (g), cholesterol (mg) and sodium (mg) per day. Friedman's test with Dunn's multiple comparison tests were compared the different intervention groups at baseline (Day 0). The

consumption of the dietary energy and nutrients were analysed with one-way ANOVA mixed-effects analysis. For comparison of changes of major bacteria, abundances of *Bacteroides* and *Bifidobacterium* and Alpha diversity from Day 2 to Day 0 for the three spices intervention groups, Friedman with Dunn's multiple comparison tests were performed. Paired individual changes of above two bacteria were analysed by Wilcoxon matched-pairs signed rank test. A non-parametric Spearman rho and two-tailed p values were calculated for the correlation between changes of three metabolites and relative abundance of bacterial genera. The changes in the relative abundances of two bacteria (*Bacteroides* and *Bifidobacterium*) in relation to their absolute abundance at baseline (Day 0) for the various curry doses (D0C, D1C and D2C) were analysed using simple linear regression. The comparison of relative abundance of major bacteria at baseline were provided and described as Mean ± SD and the intra-individual variation at baseline between the three doses is presented as mean % Coefficient of Variation (%CV). All the statistical analyses were performed by GraphPad Prism 8 (GraphPad Software Inc., San Diego, USA).

Data availability

The relevant data are provided in the paper and the raw sequencing data can be found at the EBI repository (accession no: PRJEB35853).

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Author contributions

The author contributions are as follows: conceptualisation, S.H. and Y.K.L; methodology, S.H., W.W.T.K. and S.D.L; software, W.W.T.K.; validation, W.W.T.K., S.H. and Y.K.L.; formal analysis, W.W.T.K.; investigation, W.W,T.K and S.H.; resources, S.H., Y.K.L.; data curation, W.W.T.K and S.D.L.; writing—original draft preparation, W.W.T.K, S.H. and S.D.L.; writing—review and editing, S.H., W.W.T.K and Y.K.L; visualization, W.W.T.K and S.H.; supervision, Y.K.L. and S.H.; project administration, S.H.; funding acquisition, Y.K.L.

Competing interests

The authors declare no competing interests.

Additional information

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