The fecal microbiota of Thai school-aged children associated with demographic factors and diet

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Background. Birth delivery method and breastfeeding practices contribute to microbiota colonization. Other factors including, diet and demographic factors, structure gut microbiome assembly and diversity through childhood development. Exploration of these factors, especially in Southeast Asian children, remains limited.

Methods. We investigated fecal microbiota of 127 school-aged children in Thailand using quantitative PCR (qPCR) to assess the influence of diet and demographic factors on the gut microbiota. Multivariate analysis (multiple factor analysis (MFA) and Partial Least Squares Discriminant Analysis (PLS-DA)) were used to link particular gut microbes to diet and demographic factors.

Results. Diet and demographic factors were associated with variation among gut microbiota. The abundance of *Gammaproteobacteria* increased in children with infrequent intake of high fat foods. Obese children possessed a lower level of Firmicutes and *Ruminococcus*. *Bifidobacterium* was enriched in pre-teen aged children and detected at lower levels among formula-fed children. *Prevotella* was more abundant in children who were delivered vaginally. While ethnicity explained a small amount of variation in the gut microbiota, it nonetheless was found to be significantly associated with microbiome composition.

Conclusions. Exogenous and demographic factors associate with, and possibly drive, the assembly of the gut microbiome of an understudied population of school-aged children in Thailand.

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Abstract

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Methods. We investigated fecal microbiota of 127 school-aged children in Thailand using quantitative PCR (qPCR) to assess the influence of diet and demographic factors on the gut microbiota. Multivariate analysis (multiple factor analysis (MFA) and Partial Least Squares Discriminant Analysis (PLS-DA) were used to link particular gut microbes to diet and demographic factors.

Results. Diet and demographic factors were associated with variation among gut microbiota. The abundance of *Gammaproteobacteria* increased in children with infrequent intake of high fat foods. Obese children possessed a lower level of Firmicutes and *Ruminococcus*. *Bifidobacterium* was enriched in pre-teen aged children and detected at lower levels among formula-fed children. *Prevotella* was more abundant in children who were delivered vaginally. While ethnicity explained a small amount of variation in the gut microbiota, it nonetheless was found to be significantly associated with microbiome composition.

Conclusions. Exogenous and demographic factors associate with, and possibly drive, the assembly of the gut microbiome of an understudied population of school-aged children in Thailand.

Introduction

Microbial colonization of the gut starts upon birth, and the composition of the microbiota community diversifies throughout childhood. Proteobacteria and Actinobacteria dominate the gut microbiome early in life (Zhuang et al., 2019). As infants develop, their gut microbiota community becomes more complex and, at two to three years of age, its structure and composition begins to more closely resemble that of adults (Rodríguez et al., 2015). Shifts in the microbiota composition occur during this process and are influenced by mode of delivery, feeding type, and diet (Martin et al., 2016; Rutayisire et al., 2016; Iddrisu et al., 2021).

Early in life, the assembly of the gut microbiome highly depends on delivery mode and feeding practices (Li et al., 2020; Mitchell et al., 2020; Coker et al., 2021). The microbiome of infants born vaginally are more enriched in *Bifidobacterium* and/or *Bacteroides* compared with those infants delivered by cesarean section (Yang et al., 2019; Reyman et al., 2019; Niu et al., 2020). Over time, the association between gut microbiota and mode of delivery weakens (Rutayisire et al., 2016). Nonetheless, differences in the abundance of microbiota between children of different modes of delivery persists in school-aged children (Salminen et al., 2004). *Bifidobacterium*

78 dominates the gut of infants receiving breast milk as compared to formula fed children (van den
79 Elsen et al., 2019; Lawson et al., 2020). The impact of breastfeeding duration persists later in life
80 (Zhong et al., 2019). This suggests that delivery mode and feeding type could have a long-term
81 impact on the diversity of one's gut microbiome.
82
83 Additional host-related factors such as ethnicity, age, and body mass index (BMI) contribute to
84 alterations of gut microbiota. Geographical factors and ethnicity significantly affect gut
85 microbiota profiles of school-aged children at the genus level (Liu et al., 2020). Although many
86 studies have already monitored compositional changes in the gut microbiota depending on one's
87 age (e.g., comparing between childhood and adulthood) (Derrien, Alvarez & de Vos, 2019), data
88 on microbiota profiles among school-aged children remains limited (Odamaki et al., 2016).
89 Variation in the microbiome among children has also been linked to BMI (Bervoets et al., 2013;
90 Da Silva, Monteil & Davis, 2020; Shin & Cho, 2020). These changes have been associated with
91 diets which are precursors to weight gain and shape the gut microbiome (Voreades, Kozil &
92 Weir, 2014; Cho, 2021).
93
94 Diet can shape the human gut microbiota (Singh et al., 2017; Zmora, Suez & Elinav, 2019). In
95 children, diet explains most of the variation in gut microbiota profiles between countries or
96 continents (De Filippo et al., 2010; Nakayama et al., 2015) as already observed in adults (Yasir
97 et al., 2015; Escobar et al., 2015; Ghosh et al., 2020). Moving away from traditional diets with a
98 high concentration of fiber, fruits and vegetables towards a Western diet rich in animal protein,
99 fat and sugar is a cause of concern as high fat diets have been shown to disrupt the balance of gut
100 microbiota in animal models (Kim et al., 2012). This effect has also been observed in humans
101 where a decrease in the abundance of butyrate-producing bacteria has been noted in populations
102 consuming higher-fat diets (Wan et al., 2019). Recently Southeast Asian populations have begun
103 to adopt the Western diet (Ooraikul, Sirichote & Siripongvutikorn, 2008). However, only few
104 studies have investigated the effects of this dietary pattern on the gut microbiota of young
105 Southeast Asians (Nakayama et al., 2017; Golloso-Gubat et al., 2020).
106
107 This study is the first to examine the impact of diet and demographic factors (gender, age, BMI
108 z-score, birth records, feeding type, and ethnicity) on the gut microbiota of school-aged children
109 in Thailand. Multivariate analyses were implemented to determine the potential contribution of
110 multiple factors on variations of microbiota profiles as well as identifying most relevant features
111 (microbiota taxa) for each host variable. Our results provide a preliminary overview of the
112 associations observed between the abundance of gut microbiota and investigated factors in
113 school-aged children from Thailand.
114

Materials & Methods
116 **Ethics approval**

117 All participants provided written informed consent (Supplementary File 1) and the study was
118 approved by the Ethics committee of Mae Fah Luang University (Ethics Registry: REH-61204).
119 The study was conducted in accordance with the Declaration of Helsinki.
120
121 **Study population and group definition**
122 We recruited 127 children from Ban Huai Rai Samakee elementary school in Chiang Rai,
123 Thailand. Recruitment of subjects was conducted by voluntary participation through the school's
124 administration. Parents provided informed consent prior to participation. Demographic data
125 collection included gender, age, weight, height, ethnicity, history of birth delivery mode and
126 feeding practice (representing the feeding mode in infancy) (Supplementary File 2). The child's
127 weight and height were measured by class instructors. Information on birth delivery method and
128 feeding type were collected through child self-report and/or parental-report surveys. Body mass
129 index (BMI) derived from the weight (kg) and height (m^2) ratio was converted into gender-
130 specific z-scores for BMI-for-age according to BMI cut-offs for children (5-19 years) set by
131 World Health Organization (de Onis et al., 2007). Z-scores for BMI-for-age were classified into
132 5 groups: severe thinness (SVThinness; < -3 SD; n = 1), thinness (≥ -3 SD to < -2 SD; n = 5),
133 normal weight (≥ -2 SD to + ≤ +1.0 SD; n = 83), overweight (OV; > +1SD to ≤ +2SD; n = 20),
134 and obese (OB; > +2 SD; n = 18) (Fig. S1). Age groups were defined according to interquartile
135 range (IQR: 25%, 50%, and 75%): age_A (≤ 8.05 years; n = 32), age_B (8.05 < years < 11.06; n
136 = 61), and age_C (≥ 11.06 years; n = 34). Five ethnic groups were recorded in this study: Akha
137 (n = 39), Chinese (n = 34), Lahu (n = 5), Thai (n = 19), and Thai Yai (n = 30). Birth delivery
138 mode comprised vaginal delivery (n = 85) and cesarean section (n = 42). Feeding types were
139 categorized into three groups: breastfeeding (n = 98), formula feeding (n = 20), and mixed
140 feeding (n = 9).
141
142 **Dietary information**
143 Dietary habits of children were surveyed using a Thai short dietary behaviors screener developed
144 by *Let's Get Healthy!* for use in Thai ("LGH20 Food Behaviors Screener, Thai"; OHSU
145 Institutional Review Board protocol #3694). The screener included 20 questions that grouped
146 participants across five dietary behavior categories: Healthy eating behavior (HEB), fruits and
147 vegetables (FV), high sugar foods and beverages (HSFB), high salt foods (HSF), and high fat
148 foods (HFF) (Supplementary File 3A). Answer options measuring frequency of consumption
149 were divided into four levels: Frequently (daily), sometimes (weekly), infrequently (monthly),
150 and never. The scores for HEB and FV were assigned as 3 (daily), 2 (weekly), 1 (monthly), or 0
151 (never). The responses for HSFB, HSF, and HFF were reverse scored. Total component scores
152 (i.e., a sum score for each category) were divided into quartiles to assign levels of risk (low, low
153 to moderate, moderate to high, and high (Supplementary File 3B and 3C). Highest frequencies of
154 HEB and FV consumption would be associated with low risk, while high risk would characterize
155 children eating mostly HSFB, HSF, and HFF. The instrument screens general dietary behaviors,
156 but does not provide a quantitative assessment of portion size and frequency to permit

157   quantification of a specific food or nutrient intake. Instead, intake rankings permit categorization
158   of individuals according to overall dietary behaviors, such as healthy eating or high consumption
159   of fatty foods.
160
161   **Sample collection, DNA extraction, and quantitative PCR**
162   Fecal samples were collected from all children in sterilized containers and immediately frozen at
163   − 80 °C. Microbiota DNA was extracted from fecal samples using the innuPREP Stool DNA Kit
164   (Analytik Jena Biometra, Jena, Germany) according to the manufacturer's instructions. DNA
165   yield and purity were determined using the Take 3 Micro-Volume Plate (Biotek, Winooski, VT,
166   USA). Absolute quantification of bacteria was then conducted by qPCR using Real-Time
167   Thermal Cyclers CFX96 Touch™ (Bio-Rad, Singapore). Primers targeting microbiota 16s rRNA
168   genes used in this study are summarized in Table S1. Reactions consisted of template DNA,
169   forward and reverse primers, 1X SYBR green (2X SensiFASTTM SYBR No-ROX mix,
170   BIOLINE, UK), and nuclease-free water. The assay conditions and calculations of microbiota
171   copy numbers were performed according to previously described protocol (Chumponsuk et al.,
172   2021). The average estimates of microbiota abundance by converting CT values were expressed
173   as logarithmic copy number per gram of wet weight feces.
174
175   **Statistical analysis**
176   A sum score for dietary behaviors of children was visualized as a bar plot with ggplot2
177   (Wickham H, 2009). Association between dietary behavior was assessed using Spearman's rank
178   correlation and visualized with corrplot version 0.84 (Wei & Simko, 2017). Normality and
179   homogeneity of variance were tested by Shapiro-Wilk test and Levene's test (stats package
180   version 4.0.3) (R Core Team, 2020). Differences in the abundance of gut microbiota
181   (Supplementary File 4) between groups (dietary behaviors and demographic factors) were
182   determined by one-way ANOVA, Welch's t-test, and Kruskal-Wallis rank sum test ($p < 0.05$)
183   followed by multiple comparisons using Tukey's HSD test, pairwise t-tests, and Dunn's test with
184   Benjamini-Hochberg (BH) p-value correction (hereafter referred to as q-value) (stats package
185   version 4.0.3 (R Core Team, 2020) and FSA package version 0.8.31 (Ogle, Wheeler & Dinno,
186   2020). Association between birth delivery mode and the abundance of gut microbiota was
187   determined by permutational multivariate analysis of variance (PERMANOVA) with adjustment
188   for covariates (age and feeding type). Group dispersions based on a maximum distance were
189   measured by betadisper with 999 permutations in the R package vegan (version 2.5-6) (Oksanen
190   et al., 2016). Multiple factor analysis (MFA) was performed to evaluate the influence of host
191   variables (dietary behaviors and demographic factors) on variations of gut microbiota using
192   FactorMine R version 2.3 (Lê, Josse & Husson, 2008). Contribution of variables to the data set
193   was visualized with Factoextra version 1.0.7 (Kassambara & Mundt, 2020). To investigate the
194   most relevant features (microbiota taxa) in characterizing each host factor, Partial Least Squares-
195   Discriminant Analysis (PLS-DA) was carried out by the mixOmics package version 6.12.2
196   (Rohart et al., 2017). Canonical mode with 100 iterations was used as a parameter for classifying

197 classes (groups of samples). Receiver operating characteristic curve (ROC curve) and area under
198 the curve (AUC) were also calculated to examine the validity of supervised classification results.
199 Predicted scores of categorical outcomes were compared between one class versus the others by
200 Wilcoxon test (Rohart et al., 2017). The classification accuracy of PLS-DA models is interpreted
201 as follows: no discrimination (AUC 0.5), low discrimination (AUC 0.6 to 0.7), acceptable (AUC
202 0.7 to 0.8), excellent (AUC 0.8 to 0.9), and outstanding (AUC > 0.9) (Lobo, Jiménez-valverde &
203 Real, 2008; Mandrekar, 2010). All analyses were performed in R software version 4.3 (R Core
204 Team, 2020). A more detailed explanation of multivariate analyses is described in
205 Supplementary File 5.

206

207 **Results**
208 **Dietary behaviors**
209 The frequencies of dietary behaviors of children varied greatly in their score value (Fig. S2). To
210 determine their relationship between diet behaviors, we performed a correlation analysis based
211 on Spearman's rank correlation coefficient. After multiple testing corrections using the
212 Benjamini-Hochberg method, we found that high sugar foods and beverages consumption were
213 significantly correlated with high salty foods consumption ($rho = 0.39$, $q < 0.0001$) and high fat
214 foods ($rho = 0.25$, $q = 0.01$, Fig. S3). A positive association between high salt and high fat
215 behaviors was also detected ($rho = 0.27$, $q = 0.01$). Moreover, the fruits and vegetables
216 consumption were negatively correlated with every dietary behavior except for those associated
217 with healthy eating behaviors ($rho = 0.2$, $q = 0.04$). This healthy eating behavior was negatively
218 correlated with consumption of fatty foods ($rho = –0.23$, $q = 0.02$). Despite the strength of
219 association being considerably weak, the results identified a trend in children reporting high
220 unhealthy foods consumption (e.g., HSFB, HSF, HFF) also reporting low healthy foods
221 behaviors (HEB and FV).

222

223 **Gut microbiota associated with dietary behaviors**
224 MFA constructed by integration of dietary behaviors and abundance of gut microbiota revealed
225 variation in gut microbiota profiles of children (Supplementary File 6A). *Bacteroides* was highly
226 correlated with dimension 1 (Dim 1; $r = 0.91$, $p < 0.0001$), followed by *Gammaproteobacteria* (r
227 $= 0.90$, $p < 0.0001$) and total bacteria ($r = 0.89$, $p < 0.0001$) (Fig. 1A). Variation in the
228 abundances of these taxa was best explained by HFF behaviors, with an increasing trend in
229 microbial abundances indicated in HFF-low risk (coordinate $= 1.43$, $p = 0.02$; Fig. 1B). In Dim
230 2, the clusters were separated according to the number of individuals distributed in each diet
231 category. *Ruminococcus* ($r = –0.21$, $p = 0.02$) and *Akkermansia* ($r = –0.26$, $p < 0.01$) described
232 the distribution of HFF-low risk in Dim 3 (coordinate $= 1.83$, $p < 0.0001$) and Dim 4 (coordinate
233 $= 1.46$, $p < 0.001$), respectively (Fig. 1C). Both genera were decreased in individuals with low
234 HFF behaviors (Figure 1D). Other diet behaviors (HEB, FV, HSF, and HSFB), however, had a
235 lower coordinate on the first, third and fourth axes of the MFA factor map than HFF suggesting

236 less contribution of these dietary behaviors to the variation in gut microbiota profiles of children
237 in this study.
238
239 Further analysis of the association between gut microbiota and dietary behaviors using PLS-DA
240 also identified relevant features (i.e., microbiota taxa) in classifying dietary behaviors based on
241 the level of consumption. Total bacteria and *Gammaproteobacteria*, highly contributed to
242 discrimination of samples along component 1 (Dim 1), and strongly characterized HFF-low risk
243 (AUC = 0.81, p = 0.04, Figs. 2A, S4A). The abundances of total bacteria (p = 0.02, Fig. 2C),
244 *Gammaproteobacteria* (p < 0.0001, Fig. 2E), and *Lactobacillus* (p = 0.01, Fig. 2D) were
245 significantly different among HFF categories. After adjustment by multiple comparisons using
246 the Benjamini-Hochberg method, *Gammaproteobacteria* significantly increased in children with
247 low HFF compared to those with high HFF (q < 0.001), moderate to high risk HFF (q < 0.001),
248 and the highest HFF consumption (q = 0.03). In component 2 of PLS-DA for HFF, *Lactobacillus*
249 and *Ruminococcus* were the most discriminative bacteria in children reporting low HFF
250 consumption (AUC = 0.82, p = 0.03, Figs. 2B, S4B). However, a significant difference in the
251 abundance of *Lactobacillus* was detected between low HFF to moderate and high HFF
252 consumption after adjustment (q = 0.05, Fig. 2D). Moreover, PLS-DA for fruits and vegetables
253 (FV) consumption showed that total bacteria, *Prevotella*, *Bacteroides*, and *Faecalibacterium*
254 were the top three bacteria that separated children with high FV (FV-low risk) from those with
255 lower FV consumption (low to moderate risk and moderate to high risk FV consumption) (Fig.
256 S5A; AUC = 0.66, p = 0.01). The abundance of total bacteria was also significantly higher in
257 those reporting high FV as compared to those reporting lower FV consumption (q = 0.04, Fig.
258 S5C). Nevertheless, the classification was better in the second component where *Roseburia* and
259 *Ruminococcus* contributed to high FV consumption (Figs. S5B, S5D; AUC = 0.70, p < 0.001).
260 For high salty foods (HSF), *Faecalibacterium* characterized moderate to high HSF consumption
261 followed by *Bifidobacterium* and *Roseburia* on component 2, whereas *Lactobacillus* was
262 associated with low HSF consumption (Fig. S6; AUC = 0.70, p < 0.001). When considering
263 healthy eating behavior (HEB) and consumption of high sugar foods and beverages (HSFB), the
264 supervised analysis yielded no discrimination between classes (AUC < 0.6, p > 0.05). Regarding
265 the observed variability of individuals with different levels of dietary consumption, both MFA
266 and PLS-DA analyses suggested that the consumption of high fat foods had the highest influence
267 on the gut microbiota abundances in children.
268
269 **Associations between demographic factors and gut microbiota in children**
270 Analysis of gut microbiota with integration of six demographic factors (gender, age, BMI Z-
271 score, ethnicity, birth delivery records, and feeding type) illustrated differences of association
272 patterns with the gut microbiota among the demographic categories (Fig. 3 and Supplementary
273 File 7). The MFA explained 18.6% and 8.3% of the variance in Dim 1 and Dim 2, respectively
274 (Fig. S7A). *Bacteroides*, *Gammaproteobacteria*, and total bacteria were the top three variables
275 that described individual variation in Dim 1 (p < 0.0001, Fig. S7B). Their abundances decreased

276   in underweight (Thinness) and Thai ethnicity children, while an increasing trend contributed to
277   normal weight (Table 1, Figs. 3A, 3B). In Dim 2, *Lactobacillus* mainly described the variation of
278   individual profiles grouped by delivery mode ($R^2 = 0.37$, $p < 0.0001$), BMI z-score ($R^2 = 0.34$, p
279   < 0.0001), and age tertile ($R^2 = 0.31$, $p < 0.0001$) (Figs. 3C, 3D). Abundance of *Lactobacillus*
280   decreased in children delivered vaginally, and in those of normal weight, and oldest age (age_C)
281   but increased in those delivered by cesarean section, OB (obese), and youngest age (age_A).
282   Increased *Gammaproteobacteria* in middle age students (age_B), underweight (Thinness), and
283   Thai ethnicity characterized Dim 3 (respectively, Figs. S8A–S8C), while this bacteria was
284   decreased in Lahu ethnicity and oldest age (age_C). Variation of individuals in Dim 4 was
285   mainly described by Firmicutes and ethnicity ($R^2 = 0.45$, $p < 0.0001$): the abundance of these
286   bacteria was increased in children of Lahu and Thai ethnicity, but decreased in those of Chinese
287   and Akha ethnicity. In Dim 5, OV (increased) had a contrasting profile of *Ruminococcus* to OB
288   (decreased). A similar pattern of this bacterial genus was also described for mixed feeding
289   (increased) and formula feeding (decreased) (Fig. S8D). Considering all demographic variables
290   included in the MFA, gender had the least contribution to the variation in microbial abundances,
291   while other factors were associated with subtle differences, which may be of relevance to
292   profiling the gut microbiota in children.
293
294   **Correlation between gut microbiota and BMI z-score**
295   Comparisons of gut microbiota across BMI z-score groups showed a significant difference in the
296   abundances of Firmicutes ($p < 0.01$) and *Ruminococcus* ($p = 0.01$) (Figs. 4A–4B). After
297   adjustment by multiple comparisons, the abundance of Firmicutes and *Ruminococcus* were
298   significantly higher in students of normal weight ($q < 0.01$) and OV ($q < 0.05$) compared to
299   obese. The supervised analysis also indicated discriminations of these microbiota taxa between
300   BMI z-score groups (Fig. 4C). Normal BMI was highly associated with increased abundance of
301   *Ruminococcus* (component 1: AUC = 0.63, $p = 0.02$, Figs. 4D, S9), while low abundance of
302   Firmicutes and *Ruminococcus* in OB discriminated them from those in other groups (component
303   1: AUC = 0.68, $p = 0.02$, Figs. 4D, S9). A decreasing trend in the abundance of
304   *Gammaproteobacteria* and *Bacteroides* contributed to thinness (AUC = 0.76, $p = 0.04$, Fig. 4E),
305   however, their association was less important.
306
307   **Relation between gut microbiota abundance with age group**
308   Differences in the abundance of Firmicutes ($p = 0.05$) and *Bifidobacterium* ($p = 0.02$) were
309   detected at different age tertiles of school-aged children (Fig. S10). Significant increase in
310   Firmicutes ($q = 0.04$) was found in oldest children over 11 years of age (age_C) compared to
311   those in age_B ($8.05 < $ age $ < 11.06$ years) (Fig. 5A). Age_C also showed greater abundance of
312   *Bifidobacterium* than age_A ($q = 0.02$) and age_B ($q = 0.04$) groups (Fig. 5B). Further
313   evaluation of age-associated differences in the gut microbiota of children by PLS-DA revealed
314   certain microbiota taxa contributing to the discrimination. The PLS-DA plot displayed variations
315   in microbiota profiles according to age tertile (Fig. 5C). Feature classification indicated

316   Firmicutes, *Bacteroides*, *Roseburia*, *Prevotella*, and *Ruminococcus* as the top five more abundant
317   microbiota taxa in the oldest school children (age_C) (Fig. 5D). Of these, Firmicutes had the
318   highest contribution to age_C in component 1 (AUC = 0.62, $p = 0.03$, Fig. S11). The model
319   supports that children over 11 years of age have a higher abundance of this microbiota phylum.
320
321   **Comparison of microbiota abundance in different delivery mode**
322   In this study, we included a record of childbirth to determine its association with the gut
323   microbiota. A comparison of means between the two birth delivery modes showed no significant
324   difference in their abundance of microbiota (Fig. S12). When we performed PERMANOVA with
325   adjustment for covariates (age and feeding type; Supplementary File 8), the test indicated that
326   birth delivery mode was significantly associated with the abundance of *Prevotella* ($p = 0.03$, Fig.
327   S13A), while no influence of sample dispersions was detected ($p = 0.08$, and S13B). Further
328   analyses using PLS-DA also revealed variations of gut microbiota abundance based on birth
329   delivery mode (Fig. S13C). The enrichment of *Prevotella* in vaginal delivery was clearly
330   distinguished from that observed in those delivered by cesarean section (component 1: AUC =
331   0.69, $p < 0.001$, Figs. S13D, S13E).
332
333   **Differences in the abundance of gut microbiota of children associated with feeding type**
334   The gut microbiota profile of children varied across feeding types (Fig. S14). A comparison of
335   microbiota abundances among the three feeding types (breastfeeding, formula feeding, and
336   mixed feeding) showed significant differences in the abundance of Firmicutes and
337   *Bifidobacterium* ($p < 0.05$). Both bacterial taxa were significantly higher in mixed feeding
338   children than in those receiving formula feeding ($q < 0.05$, Figs. 6C, 6D). Abundance of
339   *Bifidobacterium* was significantly increased in children breastfed as infants compared to those
340   formula fed as infants ($q = 0.01$, Fig. 6D). We then analyzed the association between gut
341   microbiota and feeding type using PLS-DA to identify key-discriminatory microbiota taxa.
342   Although the PLS-DA components displayed overlapping clusters (Fig. 6A), several
343   differentially abundant bacteria that contributed to the variation in feeding type were indicated
344   (Fig. 6B). The classification model suggested that *Faecalibacterium* (Fig. 6E), Firmicutes,
345   *Roseburia* and *Bifidobacterium* increased following mixed feeding in component 1 (AUC = 0.60,
346    $p = 0.31$, Fig. S15A). In component 2, a similar pattern was observed for Firmicutes and
347   *Ruminococcus* (AUC = 0.71, $p = 0.03$), whereas *Gammaproteobacteria* increased in formula fed
348   children (AUC = 0.79, $p < 0.0001$) (Figs. 15B, S16A).
349
350   **The influence of gender towards gut microbiota profile in children**
351   Comparisons of the abundances of gut microbiota found no significant difference between
352   gender (Fig. S17). This factor, however, accounted for 47% of the variation in microbial
353   abundances observed in component 1 of PLS-DA plots of gender (Fig. S18A). Classification
354   models further demonstrated that *Lactobacillus*, *Gammaproteobacteria*, and *Bacteroides* were
355   the top three microbiota taxa associated with girls (Fig. S18B). Based on assessing the

356   discriminative ability of these microbiota taxa for each class (categorical variables), the test
357   indicated that the outcome had poor discrimination capacity to distinguish between classes (AUC
358   < 0.6, $p > 0.05$, Fig. S18C). The model indicated that gender did not influence the gut microbiota
359   profiles of children in this study.
360
361   **Correlation between ethnicity and gut microbiota composition**
362   No significant differences in the abundances of gut microbiota were found across ethnicity (Fig.
363   S19). When we included ethnicity in the PLS-DA, the model demonstrated the association of this
364   variable with the gut microbiota of children. While *Bacteroides* was the discriminative bacteria
365   in Lahu ethnicity, *Gammaproteobacteria* was enriched in individuals of Akha ethnicity
366   (component 1; AUC < 0.6, $p > 0.05$, Figs. S20A, S20B). However, a higher AUC value was
367   obtained in component 2, where *Akkermansia* discriminated Thai Yai from others (AUC = 0.68,
368    $p < 0.01$), while *Faecalibacterium* and *Roseburia* were the most discriminative bacteria in Akha
369   ethnicity (AUC = 0.67, $p < 0.01$, Figs. S20C, S20D). These models implied that ethnicity had a
370   slight influence on the gut microbiota of school-aged children.
371
372

373   **Discussion**
374   Our qPCR study of eleven microbiota taxa and total bacteria in the stool of 127 school-aged
375   children revealed associations with dietary behaviors and demographic factors. Supervised
376   analyses suggested that the gut microbiota profile was influenced by high fat foods consumption
377   and the demographic factors of BMI z-score, age, mode of birth delivery, method of milk
378   delivery, and ethnicity. Gender was not linked to variation in the gut microbiome in this study.
379
380   The human diet has a significant impact on the gut microbiota, as changes in the composition in
381   response to food consumption have been extensively documented (David et al., 2014; Zmora,
382   Suez & Elinav, 2019; Leeming et al., 2019). Here, we observed a significant increase in the
383   abundance of *Gammaproteobacteria* in children who scored lower on high fat foods behaviors
384   (characterized by the frequency of eating high fat foods, fried foods as well as food or dessert
385   which was cooked with coconut milk, butter, or margarine). Previous studies in animals, and an
386   in vitro model of the human gut using 16S rRNA gene sequencing, also showed that the
387   abundance of bacteria belonging to the *Gammaproteobacteria* class increased following a high-
388   fat diet consumption (Lecomte et al., 2015; Agans et al., 2018). To the best of our knowledge, a
389   similar finding has not been previously reported based on qPCR. Whether decrease in abundance
390   of *Gammaproteobacteria* is indeed associated with high fat diets in this population would require
391   additional validation using larger sized cohorts, and ideally combination of both high throughput
392   sequencing and qPCR for comparability across studies. An increased abundance of *Lactobacillus*
393   and *Ruminococcus* were also associated with lower HFF consumption. The abundances of these
394   bacteria are disturbed in animal models fed a high-fat diet (Daniel et al., 2014; Resch et al.,
395   2021), which indicates that these taxa may not grow well in the gut in the presence of high-fat

396 foods. Hence, in this study, lower reporting of HFF dietary behaviors among children perhaps
397 favors the growth of these bacteria. Furthermore, reporting of high fruits and vegetables
398 consumption appeared to be associated with total bacteria and *Prevotella* levels. These results are
399 consistent with previous studies of the influence of shifting from traditional to Western diets
400 (high-fat/low-fiber) on the gut microbiota of Asian populations. *Prevotella*-type taxa were
401 overrepresented in the gut of school-aged children in rural Thailand when compared with
402 children in urban areas, who harbored more *Bacteroides*-type bacteria. Frequency of fruit and
403 vegetable intake may therefore support different enterotypes, as was already reported for Filipino
404 children from rural Baybay as well as for Thai vegetarians (Ruengsomwong et al., 2014;
405 Nakayama et al., 2015, 2017). Outside of Asia, *Prevotella* dominates the microbiota
406 communities of rural African children consuming diets high in fiber compared to those of
407 European children (De Filippo et al., 2010). These converging findings emphasize the
408 importance of a fiber-rich food diet to colonize the gut with *Prevotella* (Kisuse et al., 2018).
409 High salty foods (HSF) intake affected the abundances of gut microbiota. Specifically, the
410 butyrate producer *Faecalibacterium* and *Lactobacillus* were differentially associated with
411 reported moderate to high salty foods and low salty foods consumption, respectively. A similar
412 contrasting profile between *Roseburia* (another butyrate-producing bacterium) and *Lactobacillus*
413 was previously shown in mice fed high- and low-salt diets. The former was enriched in mice fed
414 high-salt diet (Wang et al., 2017), while the proportion of the latter was significantly reduced
415 (Wang et al., 2017; Miranda et al., 2018). A similar finding has also been noted in humans
416 (Wilck et al., 2017). These findings suggest that high salt food consumption impacts the
417 abundance of specific gut microbiota members.
418
419 Changes in the gut microbiota profile of children have been associated with BMI status classified
420 based on both centiles (Bervoets et al., 2013) and z-scores (Golloso-Gubat et al., 2020; Shin &
421 Cho, 2020). In this study, a low abundance of Firmicutes and *Ruminococcus* was associated with
422 obesity, while normal and overweight children had a high abundance of these bacteria. These
423 findings are in contrast to previous studies based on 16S rRNA sequencing, whereby obese
424 children had a high abundance of Firmicutes (Da Silva, Monteil & Davis, 2020), while
425 *Ruminococcus* was nearly depleted in overweight/obese when compared to normal-weight
426 children (Karvonen et al., 2019). A longitudinal study conducted in school-aged children with
427 dietary records also highlighted a decrease of *Ruminococcaceae* in children who developed
428 obesity and had a high calorie intake (high carbohydrate/high fat and high protein/high fat)
429 associated with the obese status, (Rampelli et al., 2018). These findings suggest food intake and
430 weight gain could contribute to variability in the gut microbiome (Rampelli et al., 2018). Despite
431 unequal sample sizes and a different dietary assessment method herein, most obese children
432 (72%) consumed high fat foods quite frequently (moderate to high risk) (Fig. S21), while only
433 33% ate fruits and vegetables (Fig. S22). Thus, the observed differences in microbiota abundance
434 in our study were likely influenced by high-calorie diets, although further study with more

435 participants, longer follow-up periods, and more extensive microbiome profiling is needed to
436 verify this hypothesis.
437
438 The abundance of *Bifidobacterium* can vary across the stages of life (Arboleya et al., 2016;
439 Saturio et al., 2021) and this genus is often enriched in the gut microbiota of children (Derrien et
440 al., 2019). A similar trend was also detected in our study with a high level of *Bifidobacterium*
441 among school-aged children grouped by age tertile. The abundance of *Bifidobacterium* was
442 significantly high in children aged over 11 years. Moreover, children in previous studies that fell
443 into the same age categories as in this study also had a higher fecal concentration of
444 *Bifidobacterium* compared to those that were older aged (Agans et al., 2011; Hollister et al.,
445 2015; Zhong et al., 2019). Concerning age variables, a gap may exist with these findings as we
446 stratified individuals by quantile ranges. Whether or not the presence of this particular bacterium
447 is associated with age, changes in *Bifidobacterium* levels from childhood to adolescence using
448 narrow-age ranges may be worth investigating to better comprehend this relationship.
449
450 Both birth delivery method and feeding type appears to have a strong influence on the early-life
451 gut microbiota (Cukrowska et al., 2020; Mitchell et al., 2020). The impact of the former has been
452 shown in a large longitudinal analysis of gut microbiota from 600 newborns and 175 mothers,
453 which denoted significant differences in the composition of gut microbiota between cesarean
454 section born and vaginally delivered infants (Shao et al., 2019). The latter type of birth was
455 associated with a high abundance of *Prevotella*, as shown in newborns and during the first two
456 years of life (Dominguez-Bello et al., 2010; Bokulich et al., 2016). Although our study was
457 conducted in school-aged children, enrichment of this genus was still observed in those who
458 were born vaginally. This result implies that the impact of method of delivery may continue
459 beyond infancy. Furthermore, we found that the abundance of *Bifidobacterium* was lowest in
460 children who were formula fed as infants when compared with children who were either breast
461 fed or mixed fed during infancy. *Bifidobacterium* abundance is increased in the gut of breast-fed
462 infants rather than in those that are formula-fed. It has been speculated that the bacterium utilizes
463 human milk oligosaccharides (HMO) (Lee et al., 2015; Forbes et al., 2018; Lawson et al., 2020).
464 Our data suggests that a lack of exposure to breast milk at an early age may reduce abundance of
465 gut *Bifidobacterium*, while mixed-feeding may stabilize the abundance close to breastfeeding. As
466 time progresses, however, many other factors including the influence of one's diet is expected to
467 also influence the makeup of one's gut microbiome.
468
469 Ethnicity introduces variations in the gut microbiota profiles through diet (Khine et al., 2019;
470 Dwiyanto et al., 2021). Considering the small sample size of our study, however, our findings
471 did not have an adequate power to identify the associations between the consumption of ethnic-
472 based diets and the abundance of gut microbiota. We did, however, observe a trend when
473 discriminating between ethnic groups. For instance, two genera within the phylum Firmicutes
474 (*Faecalibacterium* and *Roseburia*) were associated with children of Akha ethnicity, whereas

475 *Akkermansia* was mainly found to associate with the Thai-Yai ethnic group. These results are
476 inconclusive due to a lack of dietary data relating to ethic cultural practices.
477
478 Although our study demonstrated the independent effect of each host factor on the gut
479 microbiota, our results should be interpreted with caution. Major limitations include the lack of
480 sample size estimation and data on cultural practices (e.g., traditional diets, lifestyle, etc.). Since
481 recruitment of subjects was based on voluntary participation, the number of subcategories was
482 not homogeneous. In this regard, inter-individual variation was investigated using multivariate
483 statistical analyses with all concerned factors. The same method has been implemented in our
484 previous works (Gruneck et al., 2020; Chumponsuk et al., 2021). Moreover, we were unable to
485 collect data on cultural practices due to the language barriers, which might link to dietary
486 behaviors of these school-aged children. Both limitations described above serve to limit our
487 ability to explore correlations between important risk factors and the gut microbiome of school-
488 aged children. One such potential confounding factor, physical activity, should also be included
489 with future studies to better understand the role this plays together with BMI and diet.
490

Conclusions

492 This study highlights how diet influences gut microbiota. A high abundance of
493 *Gammaproteobacteria* was noted in children who reported consumption of few high fat foods
494 consumption. Demographic factors such as BMI z-score, age, and feeding type also
495 demonstrated their potential associations with gut microbiota. Obese children were characterized
496 by a low abundance of *Ruminococcus*. Those over 11 years of age were found to have a high
497 level of *Bifidobacterium*, whereas this abundance decreased in children with a history of formula
498 feeding. Moreover, birth mode and ethnicity displayed a trend towards the enrichment of gut
499 microbiota. Considering all host variables, gender was not a determinant of microbiota profiles
500 in this study.
501

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505

Abbreviations

507 HEB – Healthy eating behavior
508 FV – fruits and vegetables
509 HSFB – high sugar foods and beverages
510 HSF – high salt foods
511 HFF – high fat foods
512 OV – overweight
513 OB – obese
514 age_A – age of children ≤ 8.05 years

515 age_B – age of children between 8.05 and 11.06 years (8.05 < years < 11.06)
516 age_C – age of children ≥ 11.06 years

517

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758

Gut microbiota and categorical variables (demographic factors) that most described variation of individuals in each dimension obtained by the MFA

1 **Table 1** Gut microbiota and categorical variables (demographic factors) that most described
2 variation of individuals in each dimension obtained by the MFA

Dimension	Dimension described by quantitative variable (bacterial taxon)	Correlation with the dimension (r); p-value[a]	Dimension described by categorical variable	[b]Coordinate; p-value[c]
1	*Bacteroides* *Gammaproteobacteria* total bacteria	0.90; < 0.0001	Thinness (BMI z-score)	−1.62; < 0.01
		0.88; < 0.0001	Thai ethnicity	−1.29; < 0.001
		0.85; < 0.0001	Normal weight (BMI z-score)	1.19; $p < 0.001$
2	*Lactobacillus*	0.26; < 0.01	Vaginal delivery	−0.81; $p < 0.0001$
			Normal weight (BMI z-score)	−0.77; $p < 0.0001$
			[d]Age_C	−0.76, $p < 0.001$
			Cesarean section	0.81; $p < 0.001$
			OB (BMI z-score)	1.29; $p < 0.0001$
			[d]Age_A	1.06; $p < 0.0001$
3	*Gammaproteobacteria*	0.21; 0.02	[d]Age_B	1.00; $p < 0.0001$
			Thinness (BMI z-score)	2.03; $p < 0.0001$
			Thai ethnicity	1.58; $p < 0.0001$
			Lahu ethnicity	−2.45; $p < 0.0001$
			[d]Age_C	−0.92; $p < 0.0001$
4	Firmicutes	0.29; 0.01	Lahu ethnicity	1.59; $p < 0.0001$
			Thai ethnicity	0.85; $p < 0.0001$
			Chinese ethnicity	−1.08; $p < 0.001$
			Akha ethnicity	−0.97; $p < 0.01$
5	*Ruminococcus*	0.29; < 0.0001	OV (BMI z-score)	1.16; $p < 0.0001$
			OB (BMI z-score)	−1.07; $p < 0.01$
			Mixed feeding	1.02, $p < 0.01$
			Formula feeding	−1.21; $p < 0.0001$

3 [a]An F-test was used to assess whether the variable had a significant influence on the dimension. [b]A positive value
4 indicates an increasing trend, while a negative value represents a decreasing trend. [c]A t-test was done to see whether
5 the coordinates of the individuals in one category are significantly different from others. [d]Age tertile (corresponding
6 to 25 %, 50 %, and 75 %); age_A ≤ 8.05 years, age_B 8.05 < age < 11.06 years, age_C ≥ 11.06 years.

Figure 1

Multiple factor analysis (MFA) of the integration of gut microbiota and dietary behaviors of school-aged children.

The correlation circle plot showing the correlation between quantitative variables (microbiota taxa at the phylum, class, and genus levels) and dimensions (A: Dim 1 and 2, C: Dim 3 and 4). A variable that is close to the circle is highly correlated to the dimension. (B) The factor map of individual profiles grouped by high fat foods (HFF) consumption in Dim 1 and 2. (D) The factor map of individual profiles grouped by high fat foods (HFF) consumption in Dim 3 and 4. The categorical variables were specified by the 95% confidence ellipses.
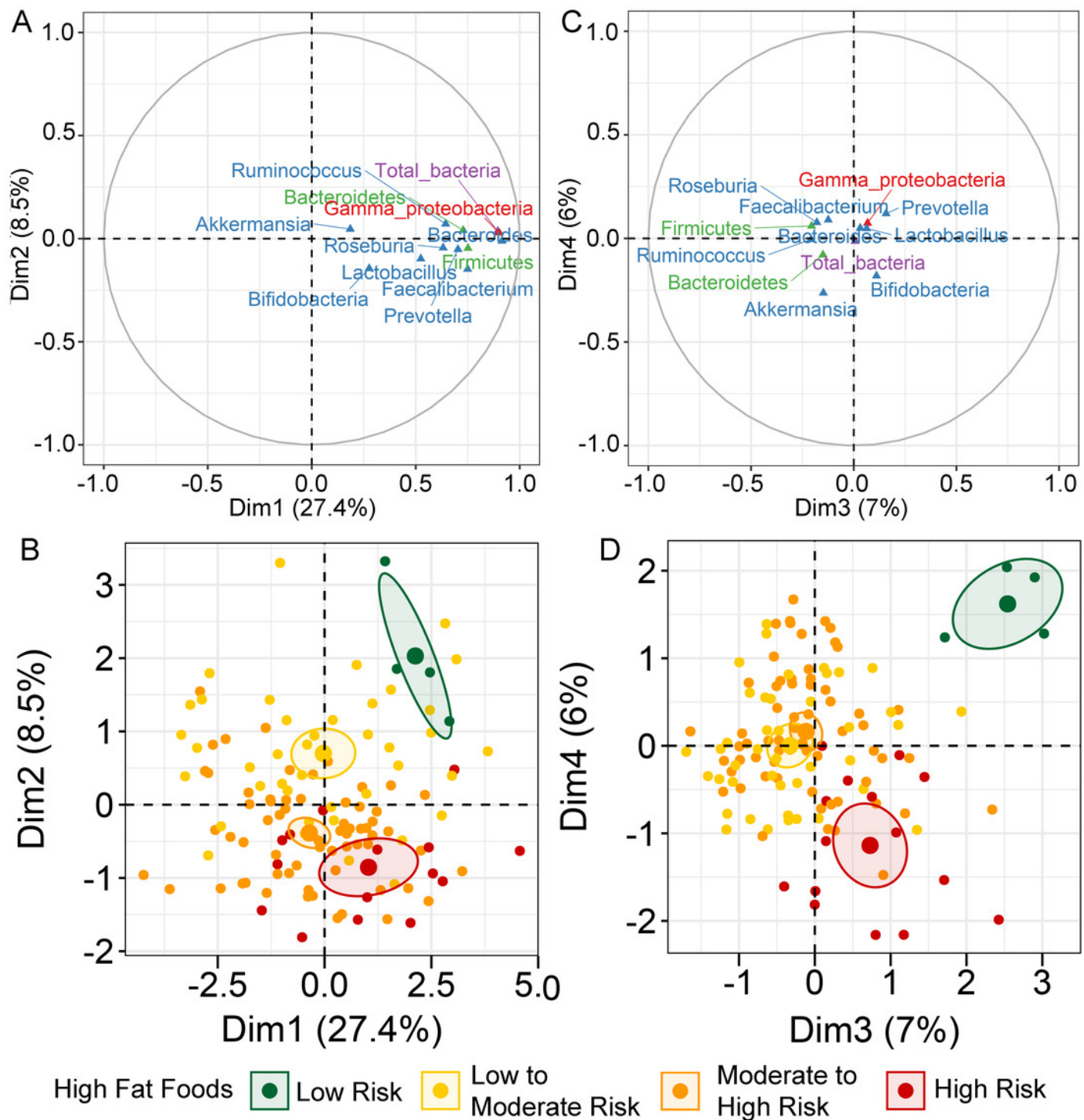
Figure 2

Partial least squares discriminant analysis (PLS-DA) of gut microbiota in school-aged children with high fat foods (HFF) consumption.

Discriminant analysis demonstrating variable selection (microbiota taxa) for which the median (method = 'median') is maximum in component 1 (A) and component 2 (B). Horizontal bars indicate each bacterial taxon assigned to HFF consumption and their length corresponds to the loading weight. The importance of the bacteria contributing to the dimension runs from bottom to top. (C–E) Boxplots showing normalized bacterial abundances based on log10 qPCR 16S rRNA copy number per gram of feces. Asterisks indicates a significant difference in bacterial abundance among HFF consumption (***$q < 0.001$, *$q < 0.05$, Tukey's HSD test and pairwise t-tests with Benjamini-Hochberg p-value correction method).
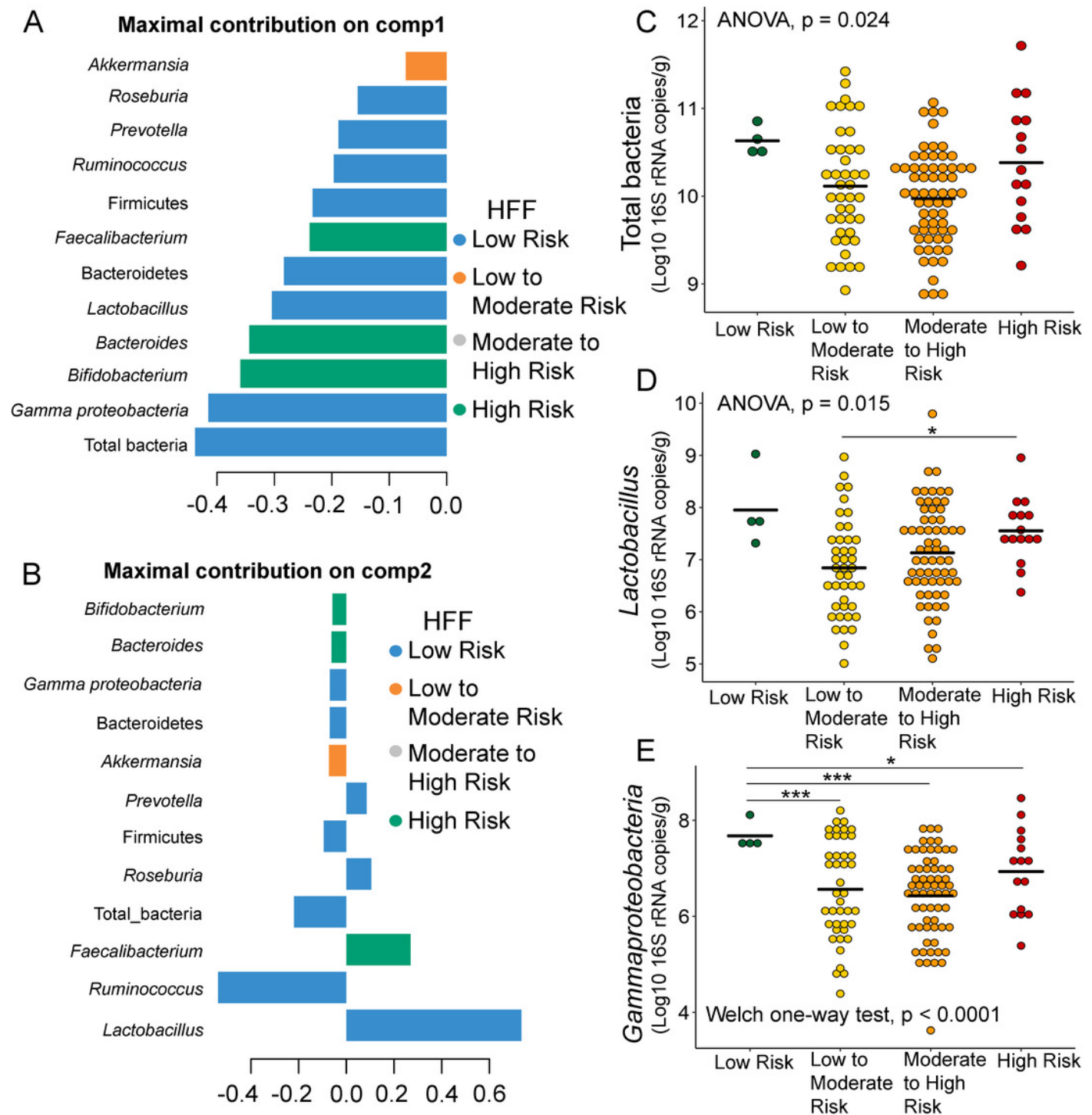
Figure 3

Multiple factor analysis (MFA) of the integration of gut microbiota in school-aged children and demographic factors.

The factor map of individual profiles grouped by BMI z-score (A), ethnicity (B), age tertile (corresponding to 25 %, 50 %, and 75 %); age_A ≤ 8.05 years, age_B 8.05 < age < 11.06 years, age_C ≥ 11.06 years (C), and birth delivery mode (D). Individual variables were specified by the 95% confidence ellipses.
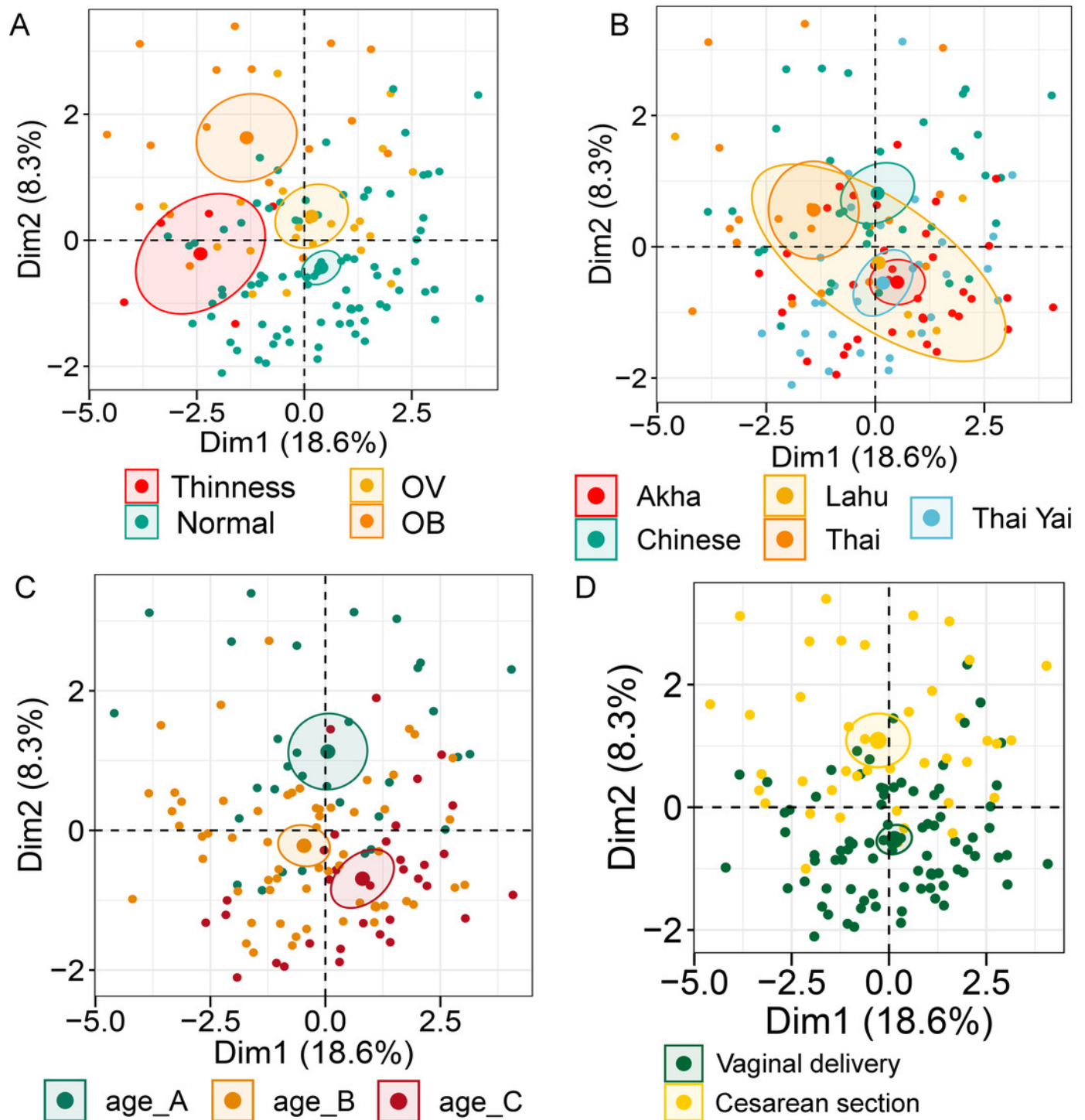
Figure 4

Partial least squares discriminant analysis (PLS-DA) of gut microbiota in school-aged children with different BMI z-score groups.

(A–B) Boxplots showing normalized bacterial abundances based on log10 qPCR 16S rRNA copy number per gram of feces. Significant differences in the abundance of *Firmicutes* and *Ruminococcus* were found between BMI z-score groups after adjusting *p*-values for multiple comparisons ($q < 0.05$, Tukey's HSD test and Dunn's test with Benjamini-Hochberg *p*-value correction method). (C) The sample plot represents variations in gut bacterial profiles of school-aged children with different BMI z-score groups (95% confidence ellipses). An explained variance was based on X-variate (normalized bacterial abundances). (D–E) Discriminant analysis demonstrating variable selection (microbiota taxa) for which the median (method = 'median') is maximum in component 1 and 2 of the sample plot. Horizontal bars indicate each bacterial taxon assigned to BMI z-score levels and their length corresponds to the loading weight. The importance of the bacteria contributing to the dimension runs from the bottom to the top of the figure. OV = overweight, OB = obese. BMI z-score cut-off points were based on WHO Multicentre Growth Reference Study Group (2006); SVThinness (severe thinness) < -3SD, Thinness ≥ -3SD to < -2SD, Normal ≥ -2SD to ≤ +1SD, OV (overweight) > +1SD to ≤ +2SD, OB (obese) > +2SD.
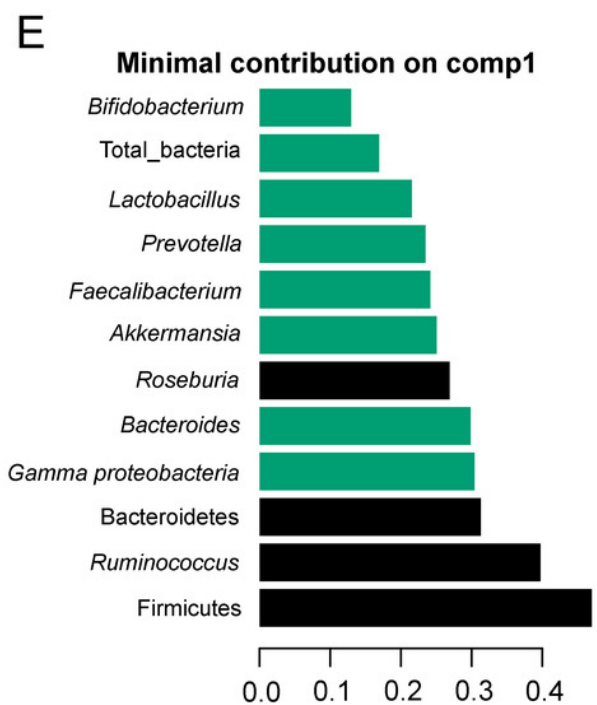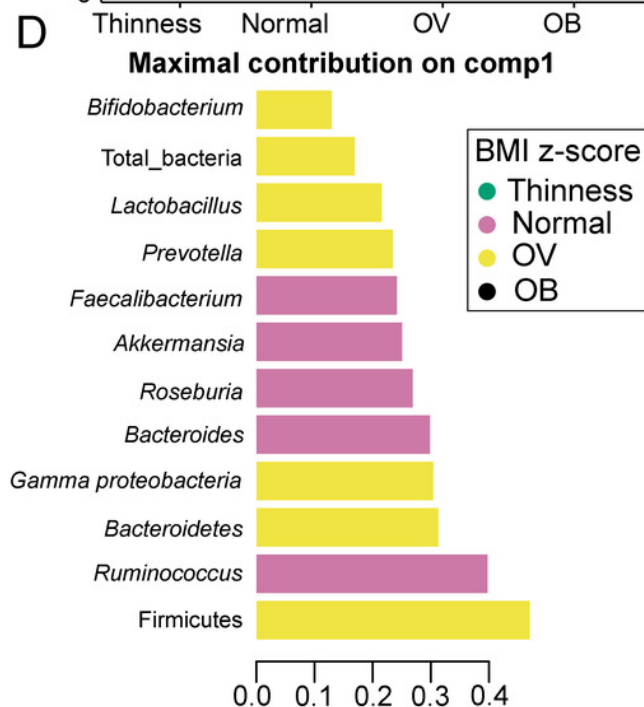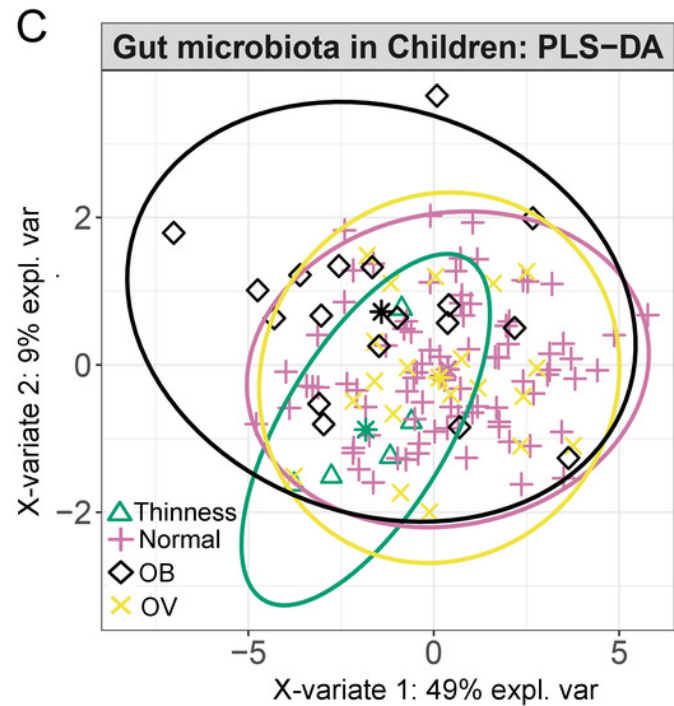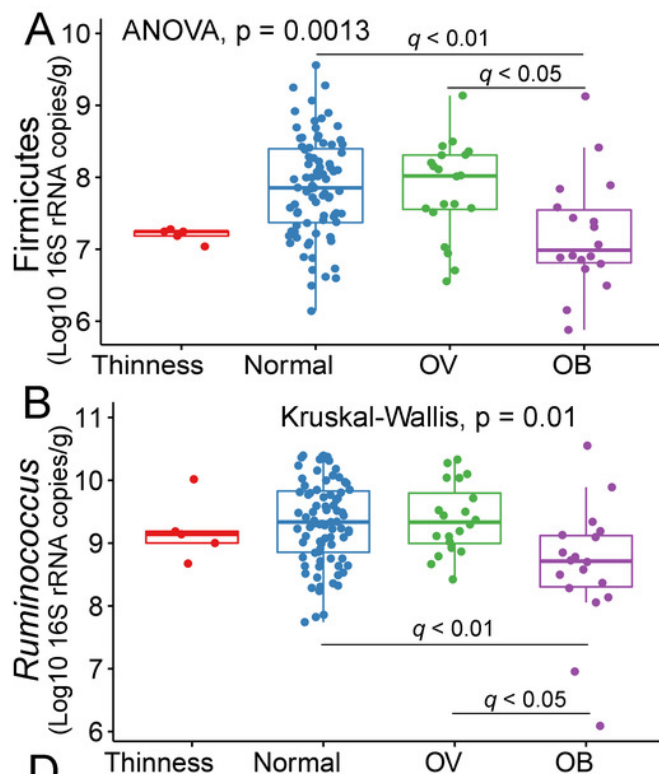
Figure 5

Partial least squares discriminant analysis (PLS-DA) of gut microbiota in school-aged children with different age tertile (corresponding to 25 %, 50 %, and 75 %); age_A $\leq$ 8.05 years, age_B 8.05 < age < 11.06 years, age_C $\geq$ 11.06 years.

(A–B) Boxplots showing normalized bacterial abundances based on log10 qPCR 16S rRNA copy number per gram of feces. Asterisks indicate a significant difference in microbiota abundance among feeding types (*q < 0.05, Tukey's HSD test and Dunn's test with Benjamini-Hochberg p-value correction method). (C) The sample plot represents variations in gut microbiota profiles of children with different age tertile (95% confidence ellipses). An explained variance was based on X-variate (normalized bacterial abundances). (D) Discriminant analysis demonstrating variable selection (microbiota taxa) for which the median (method = 'median') is maximum in component 1 of the sample plot. Horizontal bars indicate each bacterial taxon assigned to age tertile and their length corresponds to the loading weight. The importance of the bacteria contributing to the dimension runs from the bottom to the top of the figure.

A

Anova, p = 0.047

Firmicutes
(Log10 16S rRNA copies/g)

age_A   age_B   age_C

B

Kruskal–Wallis, p = 0.017

Bifidobacterium
(Log10 16S rRNA copies/g)

age_A   age_B   age_C

C

Gut microbiota in Children: PLS–DA

X–variate 2: 7% expl. var

X–variate 1: 50% expl. var

○ age_A
△ age_B
+ age_C

D

Maximal contribution on comp 1

Akkermansia
Lactobacillus
Bifidobacterium
Bacteroidetes
Total_bacteria
Gamma proteobacteria
Faecalibacterium
Ruminococcus
Prevotella
Roseburia
Bacteroides
Firmicutes

0.0   0.1   0.2   0.3   0.4   0.5

Age tertile
● age_A
● age_B
● age_C
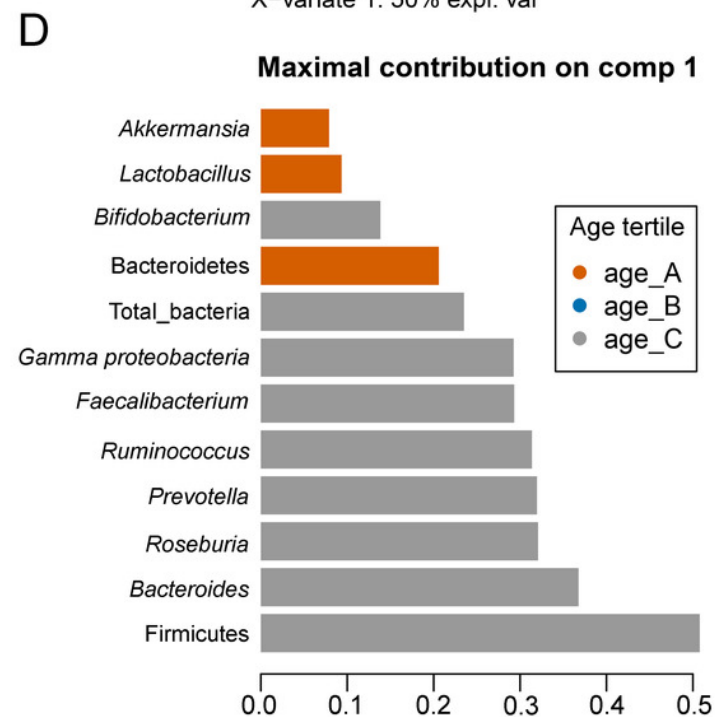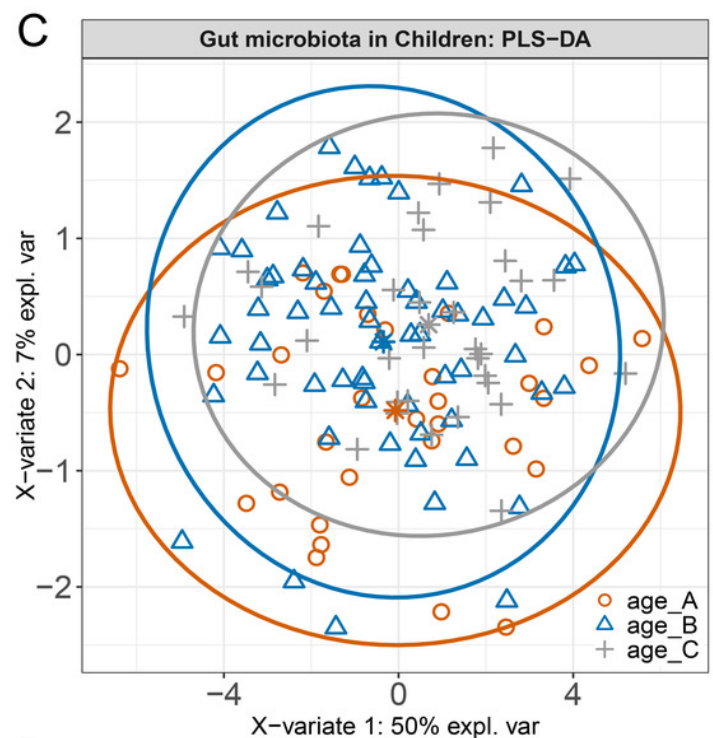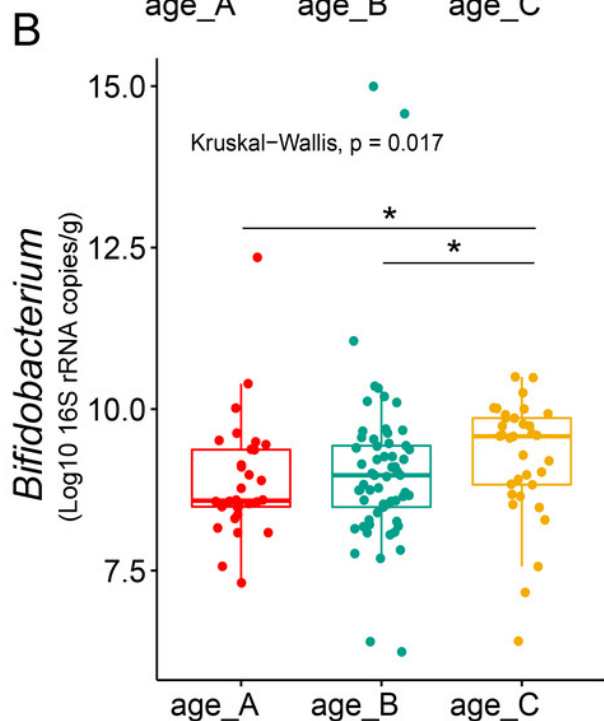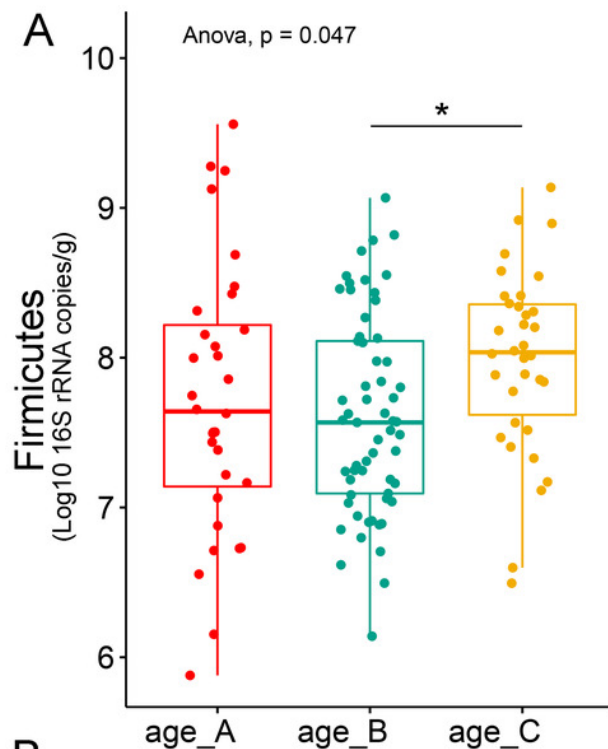
Figure 6

Partial least squares discriminant analysis (PLS-DA) of gut microbiota in school-aged children with different feeding types (representing the feeding mode in infancy).

(A) The sample plot represents variations in gut microbiota profiles of school-aged children with different feeding types (95% confidence ellipses). An explained variance was based on X-variate (normalized bacterial abundances). (B) Discriminant analysis demonstrating variable selection (bacterial taxa) for which the median (method = 'median') is maximum in component 1 of the sample plot. Horizontal bars indicate each bacterial taxon assigned to feeding type and their length corresponds to the loading weight. The importance of the bacteria contributing to the dimension runs from the bottom to the top of the figure. (C–E) Boxplots showing normalized bacterial abundances based on log10 qPCR 16S rRNA copy number per gram of feces. Asterisks indicates a significant difference in bacterial abundance among feeding types (**$q < 0.01$, *$q < 0.05$, Tukey's HSD test and Dunn's test with Benjamini-Hochberg p-value correction method).