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## Characterization of the salivary microbiome in healthy Thai children

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### ABSTRACT

**Objective:** To investigate the composition of the salivary microbiome of 50 healthy Thai children.

**Methods:** A total 76 provinces in Thailand are grouped into 5 geographical clusters based on unique economics, foods and lifestyles. Geographical locations and the results of an oral assessment were also considered. Genomic DNA was extracted from stimulated saliva samples. Subsequently, amplicon libraries were prepared by 16S Metagenomic Sequencing Library Preparation. The amplicons were sequenced using an Illumina Miseq platform followed by bioinformatics and statistical analyses.

**Results:** The correlation between oral hygiene status and caries history varied from  $r^2=0.887$  to  $r^2=0.999$  in the geographical groups, suggesting oral hygiene status a strong association between caries history. Twenty taxonomic groups were found in all subjects and constituted 93.6%–96.5% of the microbiome. Of these, genus *Veillonella* and *Prevotella* showed significant differences in their proportions between the geographical groups ( $P<0.05$ ). Furthermore, the proportion of *Veillonella parvula*, as well as *Rothia aeria* and *Rothia dentocariosa* tended to increase with worse oral hygiene status, which was also related to higher dental caries history.

**Conclusions:** The differences in the salivary microbiome as related to geographic regions suggest that environmental factors, which may include dietary habits, could influence the predominant bacteria found in the mouth of Thai children, especially the genus *Veillonella* and *Prevotella*. The ratio of *Veillonella parvula*, *Rothia aeria* and *Rothia dentocariosa* may be indicators of worse oral hygiene status and future caries in this population.

## 1. Introduction

Thailand is the 20th most populous nation in the world, with a

2015 estimate of 67 959 000 inhabitants, and the 3rd most populous in Association of South-East Asian Nations, after Indonesia and Vietnam[1]. Thailand is sub-divided into 76 provinces, along with the special administrative area of the capital Bangkok[1]. They are grouped into 5 geographical clusters, West, North, East, Central and

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South. Each region has unique economics, foods and lifestyles[1,2].

Recently, microbiome studies are accelerating our understanding of how our bodies and microorganisms interact to influence health and disease[3]. The oral microbiome plays a critical role in maintaining a physiological environment and influences oral diseases, such as dental caries[4] and periodontal diseases[5]. Furthermore, it has been reported that the composition of oral microbiome could be influenced by diet or environmental factors related to geographical location[6-8].

In this study, characteristics and composition of the salivary microbiome of 50 rural Thai children with limited access to professional dental care were investigated. Results were analyzed with respect to geographical location. Moreover, the relationship between the composition of the salivary microbiome and oral hygiene status across the 5 regions were related to the risk of dental caries.

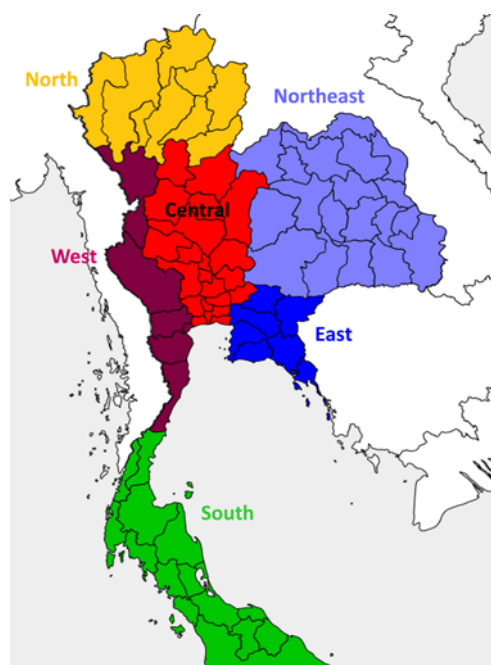
## 2. Materials and methods

### 2.1. Sample collection

All subjects, 50 Thai children, 27 males and 23 females (age range: 7 to 15 years), voluntarily joined this study under the approval from the Ethics Committee of Mahidol University with process number MU-DT/PY-IRB 2015/DT028, and saliva samples were collected on March 24th, 2015 at the Dental Hospital, Mahidol University. The participants and their parents were made aware of the objectives and procedures of the study and agreed to participate by providing written informed consent.

The teachers randomly selected these participants from Border Patrol Police Schools in Thailand after announcement of the project called “Youth Tooth Ambassador”. All 76 provinces of Thailand were geographically grouped to 5 clusters, West, North, East, Central and South, in this study (Figure 1). The participants grouped on these 5 regions received the following oral examination.

After collecting medical history of individual child from parents, children with a history of immunosuppression or systemic diseases (e.g. diabetes and HIV), use of medications that reduce saliva flow, or exposure to antimicrobials in the previous 3 months were excluded from the study. Furthermore, it was informed that subjects have to refrain from eating, drinking or cleaning their teeth 2 h before their examination. Dental caries experience was measured by using decayed, missing and filled teeth index (DMFT) for permanent teeth/dmft (for primary teeth). Six calibrated dentists examined the children for the Simplified Oral Hygiene Index (OHI-S)[9] and for dental caries using WHO criteria[10]. Ten trial patients were screened by all dentists who served as a gold standard. After the dental examination, stimulated saliva samples were collected according to a standardized protocol[6] and then stored at -80 °C until analysis.



**Figure 1.** The broad map of Thailand. All 76 provinces of Thailand were geographically grouped to 5 clusters, West, North, East (including Northeast), Central and South in this study. This map was obtained from: <https://n.freemap.jp/tp/thailand>.

### 2.2. Amplicon library construction and sequencing

Amplicon libraries were constructed by using 16S Metagenomic Sequencing Library Preparation, the V3 and V4 region, (Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System, illumina®) after bacterial DNA were extracted by the Saliva DNA Isolation Kit (NORGEN BIOTEK CORPORATION, Ontario, Canada) according to the methods described previously[11].

A quality and concentration of these amplicons were examined by Qubit® 3.0 Fluorometer (Thermo Fisher SCIENTIFIC, MA, USA) and Agilent 2200 TapeStation (Agilent Technologies, CA, USA). They were sequenced on Illumina Miseq platform using paired 300 bp reads and Miseq v3 reagents at Hokkaido System Science Co., Ltd. (Sapporo, JAPAN).

### 2.3. Data analysis

All raw sequences were processed by Cutadapt (version 1.1, <http://code.google.com/p/cutadapt/>) and Trimmomatic (version 0.32, <http://www.usadellab.org/cms/?page=trimmomatic>) with an average quality score <20. Pair-ends was concatenated by using Fastq-join (ea-utils version 1.1.2-537, <http://code.google.com/p/ea-utils/wiki/FastqJoin>), then UCLUST[12] in QIIME (version 1.8.0, <http://qiime.org/>)[13] with a 97% similarity cutoff and ChimeraSlayer[14] were used for clustering operational taxonomic units (OTUs). UCLUST algorithm with Greengenes database (version 13\_8: 16S rDNA database, [http://qiime.org/home\\_static/dataFiles.html](http://qiime.org/home_static/dataFiles.html))[15] in QIIME was used to annotate these representative sequences with a minimum identity of 90%.

### 2.4. Statistical analysis

The UniFrac metric[16] was used to determine the dissimilarity between any pair of bacterial communities. The similarity relationship, assessed using the UniFrac metric, was presented in a PCoA (Principal Coordinate Analysis) plots, drawn using QIIME. UniFrac metric cluster tree was visualized by FigTree (version 1.4.3) (<http://tree.bio.ed.ac.uk/software/figtree/>). The number of OTUs and the Shannon index (alpha diversity) were also calculated using QIIME. The Kruskal-Wallis H-test post hoc Mann-Whitney U-test with Bonferroni correction was performed to compare age, OHI-S score, DMFT, dmft,  $\alpha$ -diversity and the relative abundances of bacterial phyla and genera using ystat2008 software. Fisher’s exact test was conducted to look for differences by sex using R version 3.3.2.

$P < 0.05$  was considered statistically significant. Correlational analysis between OHI-S score and DMFT/dmft were evaluated using analysis tool in Excel 2010.

To compare differences in relative abundance at the species level, samples grouped by each oral hygiene status across 5 regions, and heat-maps were created using JAVA Treeview[17] after OTUs were clustered using Cluster 3.0 (<http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm>). OTUs with high (>2) variable importance in projection (VIP) values[18] were selected in this analysis. VIP values were calculated by every combination of salivary microbiome grouped by oral hygiene status using R mixOmics package (version 6.1.3), finally common OTUs with high VIP values in all combination were ordered.

### 2.5. Accession numbers

The obtained sequence data were deposited in DDBJ Sequence Read Archive under accession number DRA005424.

## 3. Results

A total of 50 children, 10 children from each of the 5 geographic regions, were included in this study. The demographic and oral data of the study population are shown in Table 1. Age score of West group was significantly higher than that of South group ( $P < 0.05$ ). However, this result didn’t affect further analysis in this study. Subjects grouped according to OHI-S status were shown in Table 1.

**Table 1.** General and oral condition of 50 subjects in West, North, East, Central, and South Thailand.

Index	West	North	East	Central	South
Age years	11.70±0.94	10.70±2.00	10.30±0.95	11.10±1.52	10.00±0.94
Female sex, n (%)	5(50.0)	2 (20.0)	5(50.0)	5(50.0)	6(60.0)
OHI-S	3.00±0.59	2.95±0.53	2.16±1.25	2.61±1.18	2.64±1.73
Good, n	5	3	2	3	2
Moderate, n	3	4	5	5	4
Poor, n	2	3	3	2	4
DMFT	1.50±1.58	1.90±1.37	1.30±1.41	1.20±1.48	1.40±1.35
dmft	0.90±1.29	2.00±2.10	1.30±1.25	1.10±1.37	0.90±1.10

Data are expressed as mean±standard deviations. DMFT: decayed, missing and filled teeth index for permanent; dmft: decayed, missing and filled teeth index for primary teeth; OHI-S: Simplified Oral Hygiene Index.

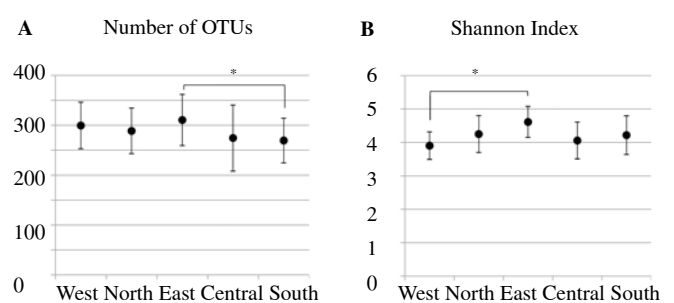
Correlational analysis between OHI-S score and DMFT or dmft in each group showed a strong correlation (West:  $r^2=0.891$  and  $r^2=0.999$ , North:  $r^2=0.887$  and  $r^2=0.996$ , East:  $r^2=0.966$  and  $r^2=0.991$ , and Central:  $r^2=0.992$  and  $r^2=0.888$ , South:  $r^2=0.898$  and  $r^2=0.998$ , respectively). This result suggested that OHI-S status was strongly associated with caries history.

Sequencing using the Illumina Miseq instrument produced a dataset comprised of 2 213 780 reads (containing the V3-V4 region), of which 1 955 658 passed quality control tests with an average length of (422±2) bp. These sequences were assigned to 21 521 OTUs defined by 97% similarity.

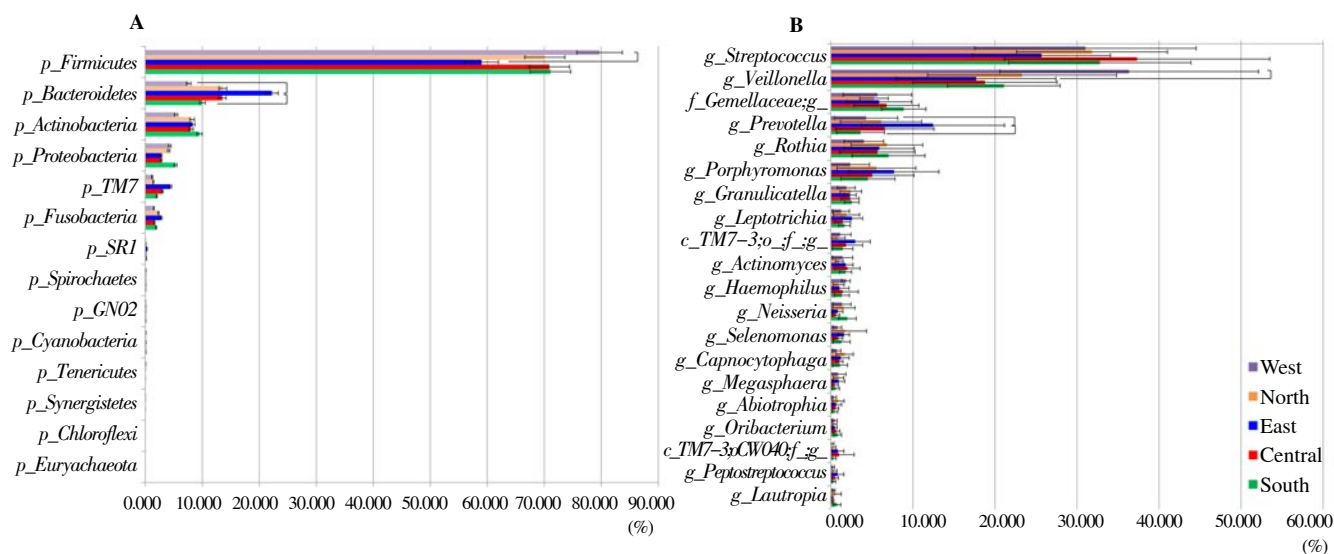
Two indices of alpha diversity indicated that salivary bacterial communities of the East group were more diverse compared to those of the South (Figure 2A) and West (Figure 2B) groups ( $P < 0.05$ ). A total 14 phyla were identified (Figure 3A). The 5 dominant phyla accounted for 99.40%-99.60% of all sequences in all 5 groups. Of these 14 phyla, phylum Firmicutes showed significant differences ( $P < 0.05$ ) between West and East group. Phylum Bacteroidetes in East group showed more predominant than other group, especially against West and South groups ( $P < 0.05$ ).

The sequences obtained in this study represented 96 genera and 63 upper-level taxa; 51 of the 96 genera and 28 of the 63 upper-level taxa and 107 species were present in the 5 groups. Twenty genera including 3 upper level taxa were found in all 50 subjects and constituted 93.6%-96.5% of the microbiome (Figure 3B). Of these 20 genera, genus *Veillonella* showed significant differences ( $P < 0.05$ ) in their proportions between the West group and the East group (Figure 3B). Furthermore, genus *Prevotella* also showed significant differences between the East group and the West & South groups (Figure 3B).

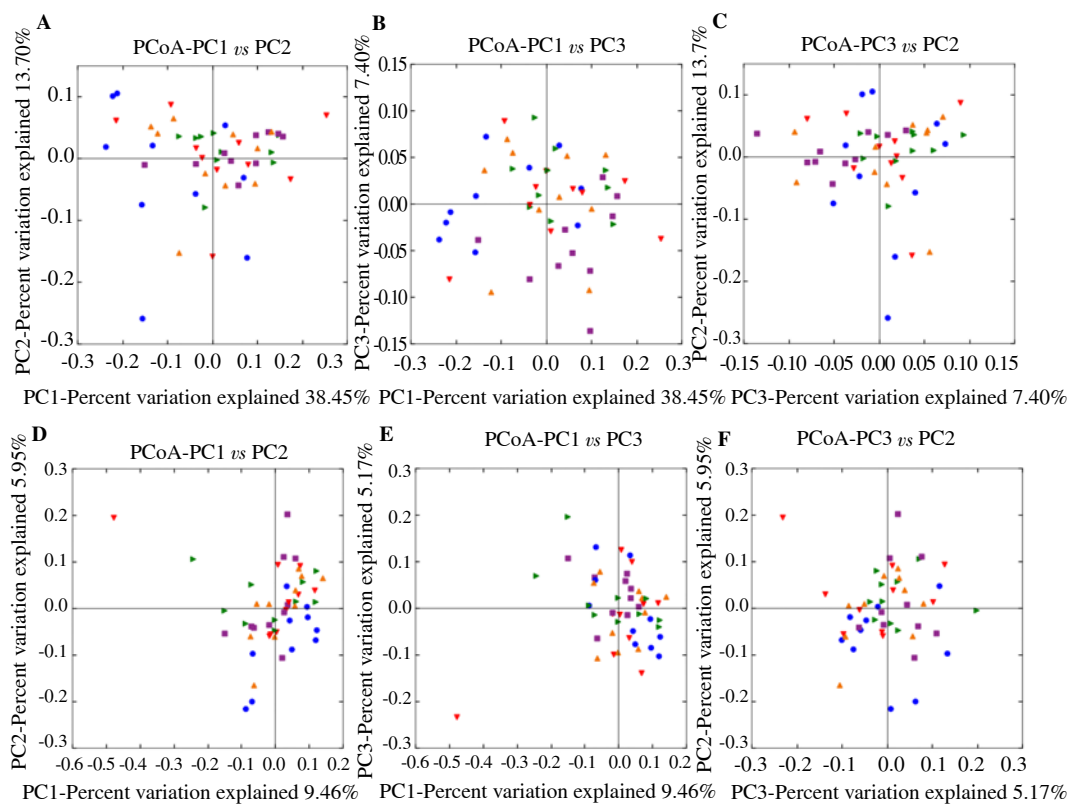
The PCoA plot based on the phylogenetic community analysis with the UniFrac metric demonstrated overall bacterial community composition similarity in each of the 5 groups (Figure 4). The PCoA plot showed a similar tendency in the distances based on the weighted (counting OTUs and the number of the reads in OTUs of each samples) versions (Figure 4A) and also unweighted (counting OTUs of each samples) versions (Figure 4B) of this metric. The differences were not confirmed statistically in both versions of this metric.



**Figure 2.** Mean number of OTUs (A) and shannon index (B) in the salivary microbiome of 50 Thai children from 5 geographical groups. The error bar indicates 95% confidence intervals. Two indices of alpha diversity indicate that salivary bacterial communities of the East group are more diverse compared to those of the South and West groups ( $P < 0.05$ ). OTUs: operational taxonomic units.



**Figure 3.** Relative abundances of all 14 bacterial phyla (A) and 20 bacterial genera including upper-taxa (B) among 5 geographical groups. *p*, *c*, *f*, and *g* indicated the phylum, class, family and genus, respectively. The error bars indicated 95% confidence intervals.

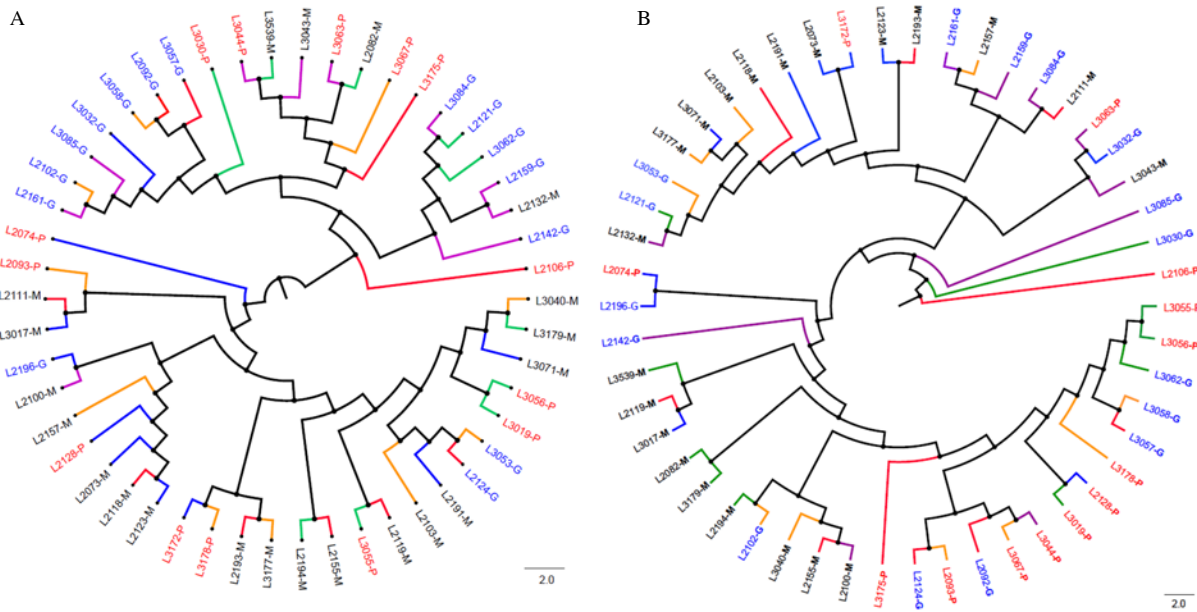


**Figure 4.** PCoA plot showing the similarity relationships among bacterial community using weighted (A-C) and unweighted (D-F) UniFrac distance metric. The purple (squares), orange (triangles), blue (circles), red (triangles), and green (triangles) indicate the West, North, East, Central, and South groups, respectively.

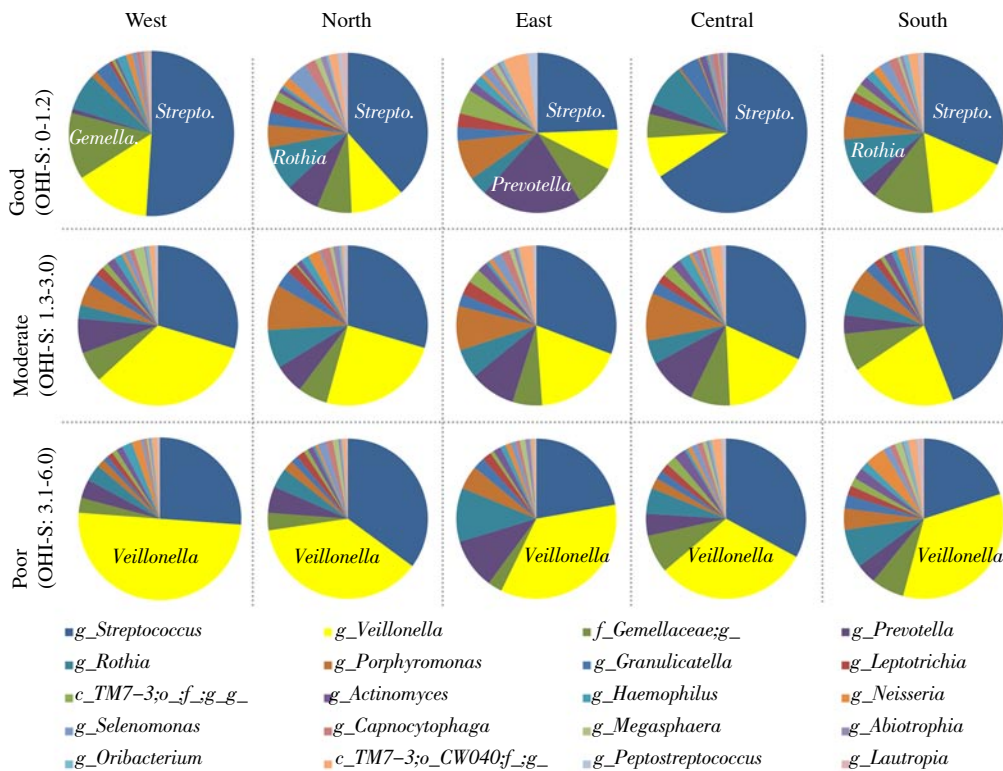
The data indicated that the microbiome of the 5 groups of subjects did not show significant differences in their community structure. According to the cluster analysis (Figure 5A and 5B), subjects that were clustered in a sub-branch have similar compositions of salivary microbiome. Both of weighted (Figure 5A) and unweighted (Figure 5B) UniFrac cluster trees showed that the microbiome of subjects in each 5 group was not similar. However, when the results of oral hygiene status (Good, Moderate and Poor) were added with each subject, almost subjects were clustered in a sub-branch across the 5

groups (Figure 5A and 5B). For example as large clusters, subject L3057, L2092, L3058, L3032, L3085, L2102 and L2161 which belonged to Good oral hygiene in weighted UniFrac cluster tree (Figure 5A) and L3539, L2119, L3017, L2082, L3179, L2194, L2102, L3040, L2155 and L2100 which belonged to Moderate oral hygiene except L2102 in unweighted UniFrac cluster tree (Figure 5B) were clustered in sub-branch with close phylogenetic distance. These findings indicated that the structure of salivary microbiome in these Thai children didn't differ by geographical location, but there were





**Figure 5.** Weighted (A) and unweighted (B) UniFrac clustering tree. Purple, orange, blue, red, and green represents the West, North, East, Central, and South group branches, respectively. Sample ID with G, M, and P showed Good, Moderate and Poor oral hygiene groups.

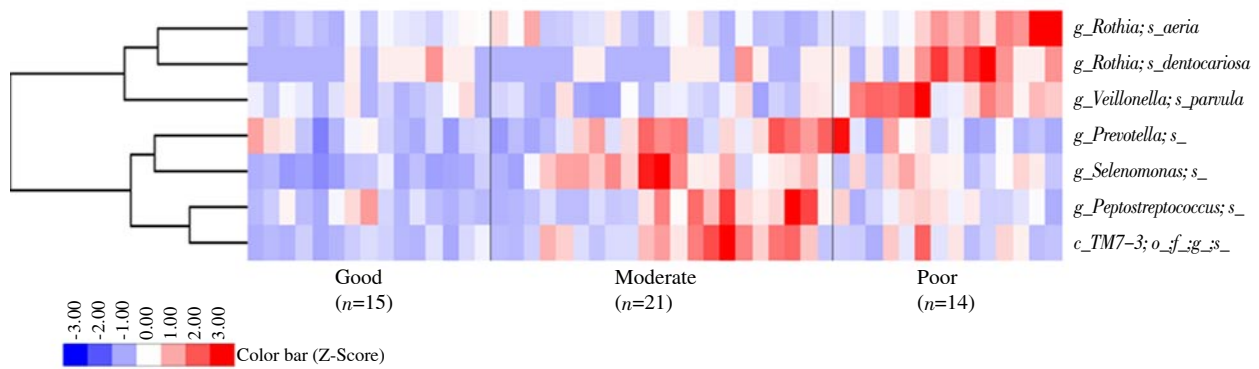


**Figure 6.** The relative abundance of 20 bacterial genera including upper-taxa in 5 geographical groups grouped by each oral hygiene status. Vertical lanes show each geographical group, West, North, East, Central and South. Horizontal lanes show oral hygiene status.

some similar microbiome compositions in each oral hygiene status.

Relative abundance of top 20 genera evidenced by the number of OTUs in each 5 clusters was examined in view of oral hygiene status (Figure 6). As the results, the proportion of genus *Veillonella* tended to increase in their salivary microbiome with worse oral hygiene status (Figure 6). In addition, the heat-map analysis was performed with the relative abundance of OTUs which had high VIP values (Figure 7). This result showed the key OTUs to characterize

each salivary microbiome grouped by oral hygiene status. 3 OTUs, which corresponded to *Rothia (R.) aeria*, *Rothia (R.) dentocariosa*, and *Veillonella (V.) parvula* were strongly associated with poor oral hygiene. Furthermore, 4 OTUs, which corresponded to unassigned species of genus *Prevotella*, genus *Selenomonas*, genus *Peptostreptococcus* and class TM7-3 were associated with moderate oral hygiene. In contrast, 0 OTU was assigned as a key OTU to characterize good oral hygiene.



**Figure 7.** Heatmap displaying species of relative abundance distribution in the three oral hygiene status across the five geographical groups. Common OTUs with VIP values >2 through all combination analysis of 3 oral hygiene groups were selected.

#### 4. Discussion

This study compared the salivary microbiome in rural Thai children with oral hygiene status, with respect to 5 geographical regions. Distinctive salivary microbiome compositions were found in different geographical locations as reported previously [7,8]. The comparison of Batwa Pygmies from Uganda and African agriculturalists from Sierra Leone and the Democratic Republic of the Congo. They suggested that their distinct lifestyle and diet (hunter-gatherer versus agricultural styles) had an impact on their salivary microbiome [7]. Takeshita *et al.* [8] found notable differences in salivary microbiome between regions having distinct diet and environmental factors (Japan and Korea). We did not find differences in composition of salivary microbiome among 5 regions in Thailand although 5 regions demonstrate different environmental factors and diet [1,2]. This inconsistency might be due to environmental factors other than, or due to the subject of children or adults, or both.

On the other hand, the present study found that the relative abundance of bacteria at the phylum and genus level showed clear significant differences between regions ( $P < 0.05$ ). Significant differences in the phylum Firmicutes and Bacteroidetes were due to those of genus *Veillonella* and *Prevotella*, respectively. Furthermore, the East group showed significant differences from the West and South group in  $\alpha$ -diversity ( $P < 0.05$ ). Potential environmental factors to account for the differences noted may include differences in the diets between regions. For example, the diets of the West and South are more similar than that of other groups. The West and South regions, which favor fermented and spicy foods similar to Malaysia [1,2]. It was suggested that these factors might affect the abundance ratio of oral bacteria. Although the determinants of the differences in the relative abundance of bacteria of salivary microbiome in this study remain unclear, the geographical differences suggest that environmental factors that differ among 5 clusters in Thailand may influence the predominant bacteria in the salivary microbiome. Gupta *et al.* [19] stated that the composition of the gut microbiome differed significantly by lifestyle among a remote hunter gatherer population, a traditional farming or fishing population and western urban industrialized population across the border of countries. From these results, the diversity

of the oral microbiome also might be influenced by such lifestyle factors. Future studies will clarify the regional differences in the oral microbiome.

Cluster analysis indicated that the structure of salivary microbiome by oral hygiene status were partially similar across the five regions under study. This observation suggests that the oral microbiome is not very diverse among the five regions of Thailand. However, the composition of the salivary microbiome may differ with respect to oral hygiene status across the 5 regions.

Thai children in rural areas especially along the country border have limited access to dental care [20]. According to the recent National Oral Health Survey, 41.9% of 12-year-old children in rural areas reported having dental pain, and 7% reported missing school days due to dental pain during the past year [21]. While the prevalence of dental caries, which is considered the main cause of dental pain in children, is lesser in the city and urban areas [20]. It was suggested that sporadic access to dental care might increase the risk for dental caries.

Moreover, the relative abundance of genus *Veillonella* especially *V. parvula*, *R. aeria* and *R. dentocariosa* tended to increase with worse oral hygiene status. Recently, Jiang *et al.* [22] reported that there was no significant differences in the salivary microbiome diversity between caries-affected and caries-free children, but the relative abundance of several species, such as *R. dentocariosa*, *Actinomyces graevenitzi*, *Veillonella* sp. oral taxon 780, *Prevotella salivae* and *Streptococcus mutans*, was higher in the caries affected group than caries-free group ( $P < 0.05$ ). Similarly, at the genus level, previous studies have indicated that *Streptococcus*, *Veillonella*, *Actinomyces*, and *Leptotrichia* accounted for a large proportion of the microbiome in caries-affected subjects [23,24]. Furthermore, at the species level, *Veillonella* HOT 780 and *Porphyromonas* HOT 284 were 4.6- and 9.0- fold higher respectively in severe early childhood caries-affected group than the caries free group [25]. It is known that the most common oral infectious disease of children is dental caries, partly resulting from poor oral hygiene. Also in this study, OHI-S status was strongly associated with caries history in every 5 clusters. The present results partially support these previous reports [22–25]. In addition, the West group had the worst 2 subjects which scored 4.34 and 4.33 in OHI-S, respectively, and the genus

*Veillonella* was significantly elevated in the West group. Therefore, the genus *Veillonella*, especially *V. parvula*, as well as *R. aeria* and *R. dentocariosa* may be useful for screening developing poor oral hygiene status that may lead to dental caries in these children. This result partially supports our previous report that the ratio of *Veillonella* in saliva tends to increase with worse oral hygiene status[11].

There are several limitations of the present study. Statistical analysis of relative abundance of oral bacteria at the genus level grouped by oral hygiene status in each 5 clusters could not be performed because of the small sample size. As the study was cross sectional, it was not possible to determine variations in the composition of the microbiome over time.

Although the salivary microbiome did not show significant  $\beta$ -diversity among 5 regions of Thailand,  $\alpha$ -diversity at the phyla and genus level was found to differ among East, West and South groups. The ratio of genus *Veillonella* increased with worse oral hygiene in all 5 regions, and the ratio of *Veillonella* especially, *V. parvula*, as well as *R. aeria* and *R. dentocariosa* may be indicators of oral hygiene status.

### Conflict of interest statement

We declare that we have no conflict of interest.

### Foundation project

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