Enteral Tube Feeding Alters the Oral Indigenous Microbiota in Elderly Adults

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Key words: oral microbiota, tube feeding, elderly, pyrosequencing, 16S rRNA
Enteral tube feeding is widely used to maintain nutrition for elderly adults with eating difficulties, but its long-term use alters the environment of the oral ecosystem. This study characterized the tongue microbiota of tube-fed elderly adults by analyzing the 16S rRNA gene. The terminal restriction fragment length polymorphism (T-RFLP) profiles of 44 tube-fed subjects were compared with those of 54 subjects fed orally (average age 86.4 ± 6.9 years). Barcoded pyrosequencing data were also obtained in a subset of the subjects from each group (15 tube-fed subjects and 16 subjects fed orally). The T-RFLP profiles demonstrated that the microbiota of the tube-fed subjects was distinct from that of the subjects fed orally (perMANOVA, $P < 0.001$). The pyrosequencing data revealed that 22 bacterial genera, including *Corynebacterium*, *Peptostreptococcus*, and *Fusobacterium*, were significantly more predominant in tube-fed subjects, whereas the dominant genera in the subjects fed orally, such as *Streptococcus* and *Veillonella*, were present in much lower proportions. Opportunistic pathogens rarely detected in the normal oral microbiota, such as *Corynebacterium striatum* and *Streptococcus agalactiae*, were often found in high proportions in tube-fed subjects. The oral indigenous microbiota is disrupted by the use of enteral feeding, allowing health-threatening bacteria to thrive.
INTRODUCTION

Enteral tube feeding is widely used to maintain nutrition in patients with a functional gastrointestinal tract, but inadequate oral intake. It is frequently used to address eating problems in frail older adults, especially those with dementia. In the United States, approximately one-third of nursing home residents with advanced dementia are tube-fed (22). Nevertheless, tube feeding in the demented elderly remains controversial. Several studies have shown that tube feeding is associated with poor survival (1, 16, 23) and an increased risk of developing pneumonia (12, 15, 28).

The oral indigenous microbiota exists in a state of balance with the host (7), but the long-term use of tube feeding alters the intraoral conditions. The absence of food passage results in an absence of mechanical clearance within the mouth and reduces saliva secretion. The mucosal surfaces often dry out, and dried sputum adheres to the palate. These ecological changes should affect the bacterial population of the indigenous microbiota. Aspiration pneumonia is a major cause of death in tube-fed elderly patients and it mainly involves oral contents (21). Unexpected bacteria may thrive in the disused oral cavity and threaten the lives of these patients.

Some well-known respiratory pathogenic bacteria, such as Pseudomonas aeruginosa, are isolated more frequently from the oropharynges of tube-fed older adults than from adults fed orally (17, 18, 30). However, the overall composition of the oral microbiota remains poorly understood due to its complexity. In addition, the opportunistic bacteria and typical pathogens could be critical etiologic agents in compromised elderly adults. Recently, we found that the global composition of the tongue microbiota is associated with the risk of pneumonia-related health problems in older adults using terminal-restriction fragment length polymorphism (T-RFLP) analysis of the 16S rRNA gene, which is a culture-independent community fingerprinting approach (31). The current study examined the rough composition of the...
tongue microbiota of bedridden elderly adults with T-RFLP and in a subset of the subjects in more detail using barcoded pyrosequencing. In this study, the microbiota of subjects fed enterally was compared comprehensively with that of subjects fed orally to characterize the oral indigenous microbiota of tube-fed elderly adults.
MATERIALS AND METHODS

Study population. The subjects were a subgroup of the population analyzed in our previous study (31). Of the 11 hospitals or nursing homes in the previous study, we selected two hospitals and one nursing home that had sufficient numbers of both tube-fed and orally fed bedridden patients; this study enrolled 98 bedridden elderly residents aged 65 and over (12 men, 86 women; mean age 86.4 ± 6.9 years). Forty-four subjects were fed with enteral tubes (31 by percutaneous endoscopic gastrostomy (PEG) tubes and 13 by nasogastric (NG) tubes), and 54 were fed orally. The ethics committee of Kyushu University Faculty of Dental Science approved this study and the procedure for obtaining informed consent. The clinical condition of each subject was evaluated using described criteria (31).

Sample preparation. Sample collection and DNA extraction from each sample were performed in our previous study (31). Tongue-coating samples were collected by scraping from the base to the tip of the tongue dorsum with a sterile plastic spatula (Muddler, Nihon Dixie, Yokohama, Japan) and DNA was extracted from each sample.

T-RFLP analysis. All 98 samples were examined using T-RFLP analysis. From each sample, the internal regions of 16S rRNA genes were amplified using the universal forward primer 8F (5′-AGA GTT TGA TYM TGG CTC AG-3′) labeled at the 5′-end with 6-carboxyfluorescein (6-FAM) and the universal reverse primer 806R (5′-GGA CTC TTA TCM CTA A-3′) labeled at the 5′-end with hexachlorofluorescein (HEX). PCR was performed using the KOD DNA polymerase (Toyobo, Osaka Japan), and cycling conditions of 98°C for 2 min, followed by 30 cycles of 98°C for 15 s, 60°C for 20 s, and 75°C for 30 s. The 16S rRNA gene amplicons were gel-purified using a Wizard SV gel and PCR purification kit (Promega, Madison, WI). Digestion with the restriction enzyme HaeIII, and electrophoresis were performed as described previously (31). The 98 T-RFLP profiles containing the electropherogram data were aligned using two different fluorescent dyes.
(6-FAM and HEX) per subject. The aligned T-RFLP profiles, excluding those terminal
restriction fragments (TRFs) detected in less than 10% of the subjects, were subjected to
principal components analysis (PCA) and displayed as a PCA diagram. PCA was performed
using R 2.10.0 (26). Candidate bacterial species corresponding to important TRFs were
selected based on their size from 755 oral bacterial 16S rRNA gene sequences (HOMD 16S
rRNA RefSeq Version 10.1) deposited in the Human Oral Microbiome Database (9). The
matching window was set to a MW ±660.

Barcoded pyrosequencing analysis. Pyrosequencing of the 16S rRNA gene was
performed in 15 subjects fed by tube and 16 subjects fed orally; subjects were selected
randomly from the 25 PEG tube-fed subjects and 42 subjects fed orally who had not used
antibiotics in the preceding month. For each subject, we reamplified the 16S rRNA gene
using 806R with the 454 Life Sciences adaptor B sequence (5′-CCT ATC CCC TGT GTG
CCT TGG CAG TCT CAG-3′) and 8F with the 454 Life Sciences adaptor A and
subject-specific 6-base barcode sequences (5′-CCA TCT CAT CCC TGC GTG TCT CCG
ACT CAG NNN NNN-3′). The PCR amplification was performed under the same conditions
as for the T-RFLP analysis. The proper size of amplicons was confirmed by agarose gel
electrophoresis and amplicons were gel-purified using a Wizard SV Gel and PCR Clean-Up
System (Promega) according to the manufacturer’s instructions. The DNA concentration and
quality were assessed using a NanoDrop spectrophotometer (NanoDrop Technologies,
Wilmington, DE), and equal amounts of DNA from 31 subjects were pooled together. One
microliter of the mixture was electrophoresed on an agarose gel to reconfirm the proper size
of the amplicons. Pyrosequencing was carried out on a 454 Life Sciences Genome Sequencer
FLX instrument (Roche, Basel, Switzerland) at the Dragon Genomics Center of Takara Bio
(Yokkaichi, Japan), and was used to determine the 16S rRNA gene sequences containing the
hypervariable regions V1 and V2.
Informatic analysis. The pyrosequencing reads were processed following the procedure described by Costello et al. (6), with some modification. Sequences were excluded from the analysis using a script written in PHP if they were shorter than 240 bases, or had an average quality score <25, and subsequently removed using a script written in R if they did not include correct primer sequence, had a homopolymer run >6 nt, or contained three or more ambiguous characters. The remaining sequences were assigned to each subject by examining the six-base barcode sequence. Similar sequences were clustered into operational taxonomic units (OTUs) using the complete-linkage clustering tool of the RDP pyrosequencing pipeline (5) at a distance cut off of 0.03, and the representative sequences of each cluster were selected using the Dereplicate request function. The representative sequences from each OTU were aligned using PyNAST (4) and the Greengenes database (8) using a minimum percent identity of 75%. Chimeras were removed from the representative set on the basis of identification as chimeric via Chimera Slayer (13) and verification that the putative chimera appeared in only one sample. After chimera elimination, a relaxed neighbor-joining tree was built using FastTree (25). To determine the dissimilarity between any pair of bacterial communities, we used the UniFrac metric (20) calculated by Fast UniFrac (14). UniFrac distances are based on the fraction of branch length shared between two communities within a phylogenetic tree constructed from all communities being compared. The similarity relationship assessed using the unweighted UniFrac metric was represented in a principal coordinate analysis (PCoA) plot drawn by R. The taxonomy of representative sequences was determined using the RDP classifier with a minimum support threshold of 60% and the RDP taxonomic nomenclature (down to the genus level). To detect the OTUs characteristically detected in the tube-fed subjects, we considered only OTUs containing at least 100 sequences. For each representative sequence of the OTUs, those detected in significantly higher proportions in the tube-fed group as compared with the orally fed group, the nearest-neighbor species with over 98% identity
were first searched using BLAST against 755 oral bacterial 16S rRNA gene sequences (HOMD 16S rRNA RefSeq Version 10.1) deposited in the Human Oral Microbiome Database (9). Sequences with no hits were further compared against a local database comprising 81,679 non-chimeric 16S rRNA gene sequences of “ncbi_tax_string” not deposited as ‘environmental samples’ in the Greengenes database (8).

Statistical analysis. All statistical analyses were conducted with R. Fisher’s exact test was conducted to look for differences by sex, institution, severity of dementia, coexisting conditions, denture use, the amount of tongue coating and tongue moisture. The Student’s t-test was performed to compare age and the numbers of teeth and decayed teeth. Wilcoxon’s signed-rank test was performed to compare the relative abundance of bacteria. Permutational multivariate analysis of variance (perMANOVA) with the function adonis in the vegan package was performed to test for differences in bacterial community structure among groups of samples. Statistical significance was set at $P < 0.05$. 
RESULTS

Of the 98 bedridden elderly persons in this study, 44 were fed via enteral tubes (31 by PEG tubes and 13 by NG tubes), and 54 were fed orally. Table 1 summarizes the general and oral conditions of the subjects in each group. Although significantly more men and severely demented persons were included in the tube-fed group, no statistical differences were observed in the other general conditions. The amount of tongue coating was significantly greater in the tube-fed group than in the group fed orally. No denture users were included in the tube-fed group.

The tongue microbiota composition of all 98 subjects was compared based on the T-RFLP profiles of the 16S rRNA gene. The overall profiles contained 235 distinct peaks (TRFs), 121 TRFs (F1 to F121) in the 6-FAM profiles, and 114 TRFs (R1 to R114) in the HEX profiles. To visualize the similarity of T-RFLP profiles, they were plotted in a PCA diagram of the first principal component (PC1) and the second principal component (PC2) using different dots to represent each feeding mode (Fig. 1A). These two components explained only 20.7% of the total variation. This low value represents the large diversity in the microbiota structures of bedridden elderly subjects. Their diverse T-RFLP patterns containing various uncommon TRFs might not be well explained by using only two virtual factors. The performance of two factorial PCA to explain overall microbial community variability is limited. Nevertheless, the diagram of these two primary principal components showed that the T-RFLP profiles of both PEG tube-fed subjects and NG tube-fed subjects differed greatly from those of subjects fed orally. The differences between the two groups were confirmed statistically using perMANOVA ($P < 0.001$). No significant difference was observed between PEG and NG tube feeding.
The loading plot of the first two principal components gave us some phylogenetic information on the microbiota of tube-fed subjects (Fig. 1B). TRFs with a large (>0.5 in absolute value) factor loading in the negative direction of PC1 were F6 and R62. Based on the fragment size, *Corynebacterium* or *Propionibacterium* species were selected from the oral bacterial database as candidate bacterial species corresponding to these TRFs (see Table S1 in the supplemental material). Conversely, 11 TRFs (R67, F42, F64, R40, F60, F67, R68, F44, F103, R78, and R57) had a positively large loading on PC1; they corresponded to other bacterial species, including *Prevotella* and *Veillonella*. Two TRFs with high loading in the negative direction of PC2 (F81 and R86) corresponded to *Streptococcus* or *Bacillus* species, while bacteria of the genus *Fusobacterium* and family *Peptostreptococcaceae* were assigned to four TRFs (R80, F70, F83, and R60) with positively large loading on PC2. Tube-fed subjects were localized in the negative direction of PC1 and the positive direction of PC2 (upper left area in the diagram), suggesting that their microbiota contain lower proportions of common oral bacteria such as *Streptococcus, Veillonella*, and *Prevotella* and higher proportions of other bacterial species, including *Corynebacterium* and *Fusobacterium*, compared to orally fed subjects.

To obtain more detailed phylogenetic information, barcoded pyrosequencing analysis was performed on 15 PEG tube-fed and 16 orally fed subjects. We determined 131,888 sequences, and 103,391 bacterial 16S rRNA gene sequences with an average length of 358 ± 71 bases passed quality control (Table 2). The sequences were assigned to 3,118 species-level OTUs using a cutoff distance of 0.03. The PCoA plot based on UniFrac, which is a phylogeny-based metric (20), also revealed that the overall microbiota composition in the tube-fed subjects was distinct from that in those fed orally (Fig. 2).

Although the microbiota diversity was confined largely to five phyla (*Actinobacteria, Bacteroidetes, Fusobacteria, Firmicutes*, and *Proteobacteria*) in both groups, the relative
proportions of these phyla varied greatly between the two groups. The tube-fed subjects had a significantly higher proportion of Actinobacteria and a lower proportion of Firmicutes compared to those fed orally (Fig. 3). In addition, the relative abundances of three minor phyla, Synergistetes, Tenericutes, and SR1, were significantly greater in the tube-fed group.

At the genus level, dominant bacterial genera commonly detected in the orally fed subjects, such as Veillonella and Streptococcus, were much less predominant in the tube-fed subjects (Fig. 4). Conversely, 22 minority bacterial genera in the usual oral cavity, including Corynebacterium, Peptostreptococcus and Fusobacterium, accounted for markedly higher proportions in the microbiota of tube-fed subjects (Fig. 5). Seven unclassified bacteria at the genus level (Family Flavobacteriaceae, Family Neisseriaceae, Family Pasteurellaceae, Family Synergistaceae, Family Incertae Sedis XI, Order Bacteroidales and Phylum Bacteroidetes) were also more predominant in tube-fed subjects than in subjects fed orally.

The genera Pseudomonas and Acinetobacter were detected only in the tube-fed group (one and three subjects, respectively). Staphylococcus was detected in four tube-fed subjects and one orally fed subject. Klebsiella was not detected in these subjects.

At the species level, defined as the 3% dissimilarity level, 54 OTUs in the tube-fed group were found in significantly higher proportions than in the orally fed group (see Table S2 in the supplemental material). Eight of these OTUs corresponded to bacteria species rarely detected in the oral cavity, such as Corynebacterium striatum, Streptococcus agalactiae, and Streptococcus dysgalactiae.
DISCUSSION

This study demonstrated that the oral microbiota of tube-fed older adults is distinct from that of those fed orally. Although the microbial composition varied among the subjects fed orally, the difference according to the feeding mode exceeded the interindividual differences (Figs. 1 and 2). Predominant indigenous members such as *Streptococcus* and *Veillonella* were detected in much lower proportions in the tube-fed subjects, whereas as many as 22 bacterial genera, including *Corynebacterium*, occurred in significantly higher proportions than in the orally fed subjects (Figs. 4 and 5). Bacterial species normally uncommon in the oral cavity, such as *C. striatum*, were also found in high proportions in their microbiota. This characteristic microbiota composition was observed in both the PEG- and NG-tube-fed subjects (Fig. 1), suggesting that it is not derived from biofilm formed on the feeding tube. We postulate that the normal balance of the microbiota is disrupted in the oral cavity when it is not used for eating. The long-term absence of food passage is an extremely abnormal situation for the oral indigenous microbiota. While fluid and carbohydrate supply is stopped, mechanical clearance by mastication drastically decreases. In addition to a reduction in the salivary flow, the biochemical composition of saliva in tube-fed subjects differs from that of orally fed ones (18). These ecological changes would be involved in the disruption of the indigenous microbiota.

No significant increase in typical respiratory pathogens, such as *P. aeruginosa*, was observed in this study, but the species that thrived in their microbiota could threaten the lives of frail elderly adults. The predominant *Corynebacterium* species, especially *C. striatum*, are potentially pathogenic bacteria with the ability to cause nosocomial outbreaks and respiratory colonization (24, 27). Anaerobic bacteria genera such as *Fusobacterium*, *Peptostreptococcus*, *Parvimonas*, and *Porphyromonas* are associated with pulmonary infections, such as pneumonia, lung abscesses, and empyema (2, 3, 11, 34). In contrast to the oral *Streptococcus*
species, *S. agalactiae* and Group B *Streptococcus* accounted for high proportions in the microbiota in tube-fed patients, and these cause invasive disease in elderly adults (10).

Although mealtime aspiration is averted by the use of a feeding tube, elderly adults fed by tube commonly aspirate contaminated oral secretions (12). Therefore, our results imply that tube-fed elderly adults continuously inhale unusual, more virulent bacteria into the lower respiratory tract and lungs. In addition, the disturbed balance of beneficial and detrimental bacteria in the indigenous microbiota, or dysbiosis, has recently attracted attention in the development of mucosal inflammation, including Crohn’s disease (19, 29, 32, 35). The oral dysbiosis that occurs with enteral tube feeding could be a health-threatening factor for frail elderly adults.

Although performing a randomized controlled trial would be difficult, the poor outcome of enteral feeding in elderly adults has been reported in several observational studies (1, 12, 15, 16, 23, 28). In addition, in our study, the incidence of pneumonia or fever and mortality in the following 6 months were significantly higher in the tube-fed subjects than in those fed orally, although this may have been due to differences in the baseline conditions of the two groups (data not shown). One should pay careful attention to the bacterial populations in the oral cavity with the use of feeding tubes. While the benefits of oral care in preventing pneumonia in elderly adults are well documented (33, 36), oral care is generally neglected in patients receiving tube feeding due to the erroneous impression that their oral cavities are not used. Rather, our results suggest that tube-fed patients need aggressive oral care to prevent the overgrowth of a disturbed microbiota, even if such care might be ineffective at restoring the normal microbiota.

This study was cross-sectional and thus cannot unequivocally demonstrate that feeding tube placement results in oral dysbiosis. A follow-up study would clarify the environmental
trigger of the dysbiosis associated with tube feeding and may lead to the development of a novel approach to prevent the oral dysbiosis in tube feeding.

In the present study, we used two different molecular approaches for microbiota comparison, T-RFLP and barcoded pyrosequencing. Although T-RFLP is highly effective for rapid comparisons of bacterial communities, it is unsuitable for predicting microbial community structure containing unexpected bacteria. Indeed, the high proportion of \textit{C. striatum} in tube-fed subjects was unable to be predicted by T-RFLP because \textit{C. striatum} is an uncommon bacterium in the oral cavity and thus not deposited in the database which we used (TRF size of \textit{C. striatum} corresponded to F6 and R62). In addition, overgrowth of \textit{S. agalactiae} in tube-fed subjects was masked by a decrease in the dominant oral \textit{Streptococcus} species, such as \textit{S. salivarius}, which generates TRF with the same size (F81 and R86).

Nevertheless, microbiota characteristics of tube-fed subjects predicted from the T-RFLP data were globally consistent with the results of pyrosequencing analysis. Although some limitations exist, T-RFLP is useful for comparisons of oral microbiota, especially in analyses using a large number of samples.

The oral indigenous microbiota is thought to serve as a defensive barrier against the establishment of more pathogenic bacteria (7). Our results clearly demonstrated that the oral indigenous microbiota is disrupted by the use of enteral feeding, allowing health-threatening bacteria to thrive. It is suggested that oral food intake plays an important role not only in nutrition but also in maintenance of a healthy oral indigenous microbiota that acts to prevent exogenous infection.
ACKNOWLEDGEMENTS

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REFERENCES


FIG. 1. (A) Principal components analysis (PCA) diagram showing the similarity relationships among the 98 T-RFLP profiles. The T-RFLP profiles of each subject are plotted according to feeding mode: orally (○), by percutaneous endoscopy gastrostomy (PEG) tube (▲), and by nasogastric (NG) tube (●). These two components explain 20.7% of the variance. (B) Loading plot of the first two principal components. Only 19 TRFs with large factor loading (>0.5 in absolute value) on the first or second principal component were selected and are indicated by arrows.

FIG. 2. Principal coordinate analysis (PCoA) plot showing the similarity relations among the 31 tongue microbiota compositions. Plots were generated using unweighted UniFrac distances. These two components explain 32.8% of the variance.

FIG. 3. The relative abundances of each phylum in the tongue microbiota of 31 subjects. The median percentages in the tube-fed and orally fed subjects are represented by solid and broken lines, respectively. *P*-values were calculated using Wilcoxon’s signed-rank test. *, *P* < 0.05; **, *P* < 0.01, ***, *P* < 0.001.

FIG. 4. The mean genera abundances of orally fed and tube-fed subjects. Only six genera commonly detected in the orally fed group (14 of 15 orally fed subjects) are shown.

FIG. 5. The relative abundances of 22 bacterial genera that were significantly more predominant in the tube-fed group compared to the orally fed group (*P* < 0.05). Statistical
differences were calculated using Wilcoxon’s signed-rank test.
TABLE 1. Baseline characteristics of bedridden elderly adults fed orally or by tube

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Tube-fed</th>
<th>Orally-fed</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td>(n = 44)</td>
<td>(n = 54)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>General conditions</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age — yr</td>
<td>85.1 ± 7.5</td>
<td>87.4 ± 6.3</td>
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</tr>
<tr>
<td>Female sex — no. (%)</td>
<td>35 (79.5)</td>
<td>51 (94.4)</td>
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<tr>
<td>Institution — no. (%)</td>
<td>0.06</td>
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<tr>
<td>Hospital A</td>
<td>27 (61.3)</td>
<td>40 (74.0)</td>
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<tr>
<td>Hospital B</td>
<td>14 (21.6)</td>
<td>7 (12.9)</td>
<td></td>
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<tr>
<td>Nursing home A</td>
<td>3 (6.8)</td>
<td>7 (12.9)</td>
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<tr>
<td>Dementia — no. (%)</td>
<td></td>
<td>&lt; 0.001</td>
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<tr>
<td>Mild</td>
<td>5 (11.3)</td>
<td>29 (53.7)</td>
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<tr>
<td>Severe</td>
<td>39 (88.6)</td>
<td>25 (46.2)</td>
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<tr>
<td><strong>Coexisting conditions — no. (%)</strong></td>
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<tr>
<td>Diabetes Mellitus</td>
<td>5 (11.3)</td>
<td>5 (9.2)</td>
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<td>Stroke</td>
<td>38 (86.3)</td>
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<td>Cancer</td>
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<td>Chronic gastroenteritis</td>
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<td>Cardiovascular disease</td>
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<td>Liver disease</td>
<td>6 (13.6)</td>
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<td><strong>Oral conditions</strong></td>
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<tr>
<td>Number of natural teeth</td>
<td>8.4 ± 9.3</td>
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<td>Number of decayed teeth</td>
<td>1.7 ± 3.3</td>
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<td>Amount of Tongue coating</td>
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<td>Moderate or much</td>
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<td>≥ 5.0</td>
<td>13 (29.5)</td>
<td>13 (24.0)</td>
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<td>1.0–4.9</td>
<td>14 (31.8)</td>
<td>22 (40.7)</td>
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*Plus-minus values are means ± SD.*
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<td>O16</td>
<td></td>
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</tr>
</tbody>
</table>

Average: 3189 ± 1913               3471 ± 2252
Fig. 2

- O: Orally fed (n = 16)
- ▲: Tube fed (n = 15)

PCO2 (18.6%) vs. PCO1 (22.2%)
Fig. 4

Orally fed

Tube fed

- Veillonella
- Streptococcus
- Prevotella
- Actinomyces
- Rothia
- Granulicatella
- Other genera