

# Relationship between oral conditions and the gut microbiome in healthy adults in Ishikawa prefecture, Japan

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## Abstract

Oral conditions may influence eating habits and nutrient intake, and nutrient intake in turn may affect the gut microbiome. Furthermore, the microbiome can affect the health of the host. However, few studies have addressed the above speculations. The present study was performed to examine the relationships between oral conditions, nutrient intake, and the gut microbiota in 239 participants in Ishikawa prefecture, Japan. Denture use altered the dietary fiber intake and increased the relative abundance of *Escherichia/Shigella* in the gut. However, several other oral conditions affected the gut microbiota without altering nutrient intake. Diets high in dietary fiber have been reported to decrease the relative abundance of *Escherichia/Shigella*. Therefore, this study suggested that denture use resulted in low dietary fiber intake and increased the abundance of *Escherichia/Shigella*. As these bacteria have been reported to cause inflammation in the colon and various diseases, the results suggested that changes in oral conditions may lead to deterioration of several diseases.

## KEY WORDS

Microbiome, Gut, Oral condition, Nutrient intake, Dietary fiber

## Introduction

Foods enter the body via the oral cavity; thus, it is speculated that oral conditions may affect eating habits. For example, tooth are very important for mincing and mashing foods to carry them to the digestive tract smoothly, therefore reducing the number of teeth causes inadequate occlusion and decreased masticatory forces; in fact, Kikutani *et al.* demonstrated that inadequate occlusion participants had a 3.2-fold greater malnutrition risk than the natural dentition group<sup>1)</sup>. Likewise, reducing masticatory forces results in a lower intake of protein and dietary fibers<sup>2-4)</sup>. Furthermore, reducing the number of teeth has been reported to increase mortality risk<sup>5)</sup>. Oral function training has been effective for nutritional improvement in nursing homes<sup>5)</sup>. These studies suggest that oral conditions may be associated with eating habits and nutrient intake.

The gut microbiome can affect health and disease status<sup>6, 10)</sup>. Some gut microorganisms encode proteins involved in functions that are important for health in the host, such as enzymes required for the hydrolysis of indigestible dietary compounds<sup>11-12)</sup>. If gut microbiome diversity is decreased, these nutrients are reduced, resulting in weakness and fatigue<sup>13-14)</sup>. Dysbiosis is a term for a microbial imbalance or maladaptation and is associated with various conditions including obesity, diabetes, irritable bowel syndrome, inflammatory bowel disease, depression, and cardiovascular disease<sup>6, 10)</sup>.

Several studies have also suggested that nutrient intake affects the host microbiome. The gut microbiome is associated with health, disease, and nutrient intake, and it is speculated that the intakes of dietary fiber, lipids, and proteins can alter microbiome diversity<sup>6-7)</sup>. Dietary patterns in Europe and the USA (low dietary

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fiber and high lipid levels) have a profound effect on decreasing *Bacteroides* and increasing *Firmicutes*, *Clostridioides difficile*, and *Escherichia coli*<sup>8)</sup>. The microorganisms in the gut of people with dietary patterns common to Europe and the USA produce lipopolysaccharide (LPS), which causes intestinal epithelium cell shedding and deterioration of general conditions<sup>9)</sup>. These results raise the question of whether the existence of several problems in the oral cavity leads to changes in nutrient intake, resulting in changes of the gut microbiota composition. However, few studies have comprehensively examined the oral condition, nutrient intake, and gut microbiota in humans. In the present study, we performed a medical examination that assessed oral conditions, nutrient intakes, and the structure of the gut microbiota in 239 participants in the Noto district, Ishikawa Prefecture, Japan, and determined the relationship between these factors.

## Methods

### 1. Ethical considerations

This study was performed in accordance with the principles of the Declaration of Helsinki, and the investigation protocol was approved by the Ethics Committee for Human Studies at the Kanazawa University Hospital. All patients provided written informed consent. Using the data resources of the Shika Study at Kanazawa University, we analyzed samples of the general population from the Horimatsu and Higashi-Matsuho districts, Shika-machi Ishikawa, Japan.

### 2. Data collection

We utilized data from the Shika study. The Shika study is a population-based survey that developed advanced preventive methods for lifestyle-related diseases<sup>15-16)</sup>. The participants' demographic information, including age, sex, and denture use, was collected through interviews or questionnaires. Oral conditions, which included remaining and carious teeth, dry oral conditions, gingival recession, dirt on teeth, and tongue coating, was evaluated by dentists and dental hygienists. The oral condition was determined using the Oral Health Assessment Tool (OHAT)<sup>17)</sup>. Nutrition status was assessed with a brief-type self-administered diet history questionnaire (BDHQ). The BDHQ asked subjects about the consumption frequency of 58 food and beverage items.

### 3. Collection of stool sample and DNA extraction

Stool samples were collected from 239 participants. First, fresh fecal samples were collected using clean paper (AS ONE Inc., Osaka Japan) and a plastic tube with a spatula (AS ONE Inc.). The plastic tubes were closed, and the fecal samples were transferred to the laboratory on ice. The samples were stored at -80°C until DNA extraction. The whole DNA was extracted from the fecal samples using the NucleoSpin® DNA Stool kit (Macherey-Nagel Inc., Düren Germany) in accordance with the manufacturer's instructions.

### 4. Next-generation sequencing (NGS)

The extracted DNA samples were processed for 16S rRNA gene sequencing using NGS<sup>18)</sup>. The hypervariable region 3 to 4 (V3-V4) of the 16S rRNA gene was amplified with Ex Taq® Hot Start Version (TaKaRa Bio Inc., Kusatsu, Japan) and TaKaRa PCR Thermal Cycler Dice® Gradient (TaKaRa Bio Inc.). The PCR fragments were purified with Agencourt AMPure XP magnetic beads (Beckman Coulter, Inc., Brea, CA, USA). After index PCR and purification, the concentration of the indexed fragments was measured with a Qubit® dsDNA HS Assay Kit using Qubit® 3.0 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The equimolar mixture of the products was sent to Hokkaido System Science Co., Ltd. (Sapporo, Japan) for Illumina MiSeq sequencing.

### 5. Microbiome analysis

The microbiome analysis was performed as described elsewhere<sup>18)</sup> with slight modifications. The raw pair-end sequences were filtered with Sickel (version 1.33)<sup>19)</sup> and combined with PANDAseq (version 2.11)<sup>20)</sup>. The chimeric sequences were removed using the USEARCH (version 10.0.240\_i86linux32)<sup>21)</sup> and Silva 16S rRNA database (release 132; 97\_otus.fasta)<sup>22)</sup>. Non-chimeric sequences were filtered by size (>300 bp).

The operational taxonomic unit selection, with a 97% similarity threshold from the non-chimeric sequences, was performed using the "pick\_de\_novo\_otus.py" command in Qiime (version 1.9.1)<sup>23)</sup> using the Silva 16S rRNA gene database (release 132) as a taxonomy database. Finally, the global singletons were excluded using the "filter\_otus\_from\_otu\_table.py" command in Qiime. This method cannot differentiate *Escherichia* from *Shigella*, so we described these genus as *Escherichia/Shigella*.

## 6. Statistics

R Statistical Package (version 3.5.0) was used to perform all the statistical analyzes<sup>24</sup>. Box plots were used to represent the first quartile, median, and third quartile, with the first quartile  $+1.5 \times$  interquartile range (IQR) and third quartile  $-1.5 \times$  IQR as whiskers and the outliers as points. The participants' characteristics (age, BMI) and nutrient intake (carbohydrate, protein, lipid, dietary fiber) among each oral condition group were compared using the Mann-Whitney U test. There was a significant difference in the participants' age according to denture use. Comparison between using denture group and not using denture group was adjusted for age by the non-parametric analysis of covariance (ANCOVA) using the "sm.ancova," package<sup>25</sup>. The relative abundance of each genus was compared using the paired Wald test in the "DESeq2" package<sup>26</sup>. Correlation of the relative abundance between two groups was assessed by the Spearman's rank correlation coefficient, denoted herein as  $\rho$ .

## Results

### 1. Participants' information

The participants' information is shown in Table 1. The median age of the 239 participants was 66.0 years, and they had a median of 25.0 remaining teeth.

Table 1. Participant characteristics. The table shows the participants' basic data, oral conditions, and nutrient intake (carbohydrate, protein, lipid, dietary fiber). Each value is shown as the median (IQR) or n (%).

Characteristic	Value
Age (years); median (IQR)	66.0 (55.0-70.0)
Female; n (%)	131 (53.3)
Body mass index; median (IQR)	23.0 (20.9-24.9)
Oral condition	
Remaining teeth; median (IQR)	25.0 (20.0-28.0)
Use of dentures; n (%)	91 (37.0)
Dirt of teeth; n (%)	189 (76.8)
Oral dry; n (%)	85 (34.6)
Gingival retraction; n (%)	222 (90.2)
Tongue coating; n (%)	219 (89.0)
Nutrients intake	
Carbohydrate (g/day); median (IQR)	250.3 (205.4-318.0)
Protein (g/day); median (IQR)	69.4 (53.2-82.5)
Lipid (g/day); median (IQR)	50.2 (38.9-68.0)
Diet fiber (g/day); median (IQR)	12.5 (9.0-16.6)

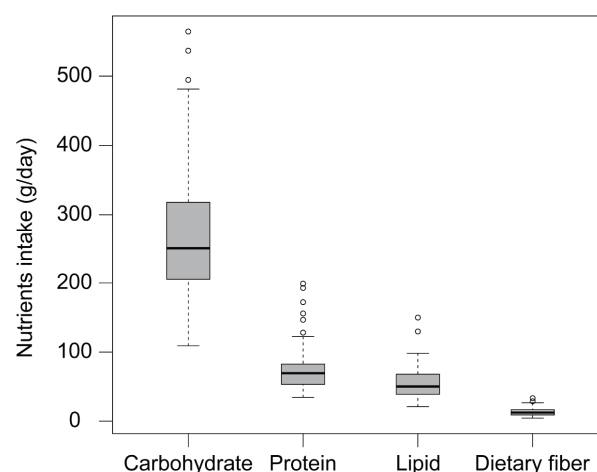


Figure 1. Nutrient intake of all participants. The box plots show the intake of each nutrient (carbohydrate, protein, lipid, dietary fiber). Median nutrient intakes: carbohydrate, 250.3 g/day; protein, 69.4 g/day; lipid, 50.2 g/day; dietary fiber, 12.5 g/day.

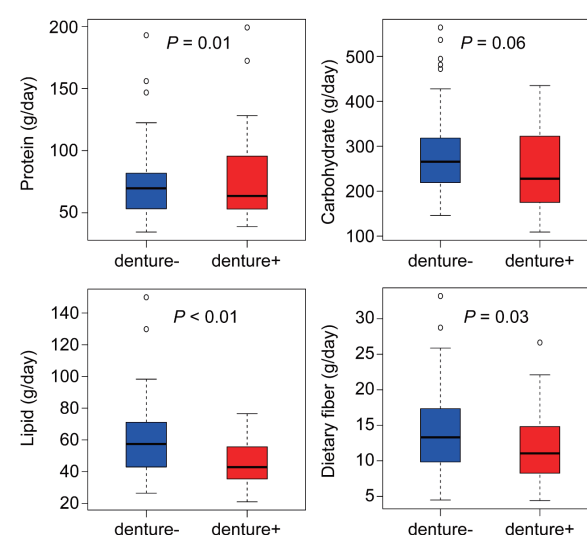


Figure 2. Comparison of nutrient intake by denture use. Each color represents a participant group: blue for no denture use and red for denture use. The  $P$  value was adjusted for age by the non-parametric ANCOVA "sm.ancova."

Females comprised 53.3% of the participants, and 37.0% of participants used dentures. The participants' nutrient intake is shown in Fig. 1. The median nutrient intakes were as follows: carbohydrate, 250.3 g/day; protein, 69.4 g/day; lipid, 50.2 g/day; dietary fiber, 12.5 g/day. There were no large differences between the "Summary of National Health and Nutrition Survey in Japan 2015" and this study (Survey in Japan vs this study: carbohydrate, 257.8 vs 250.3 g/day; protein, 69.1 vs 69.4 g/day; lipid, 57.0 vs 50.2 g/day; dietary fiber, 14.5 vs 12.5 g/day, respectively) (Ministry of Health, Labor and Welfare).

## 2. Difference of nutrient intake by oral condition

We compared nutrient intake by oral conditions (Figs. 2 and 3). Denture use was associated with decreased median protein, lipid, and dietary fiber intakes (denture- vs denture+ :protein, 73.1 vs 62.4 g/day,  $P = 0.01$ ; lipid, 57.4 vs 43.8 g/day,  $P < 0.01$ ; dietary fiber, 13.3 vs 11.1 g/day,  $P = 0.03$ ). These comparisons were adjusting for age. Although lipid intake was also increased by dry oral conditions (oral dry- vs oral dry+ :57.5 vs 48.7 g/day,  $P = 0.03$ ) (Fig. 3), there were no significant differences in nutrition intake with other oral conditions (dirt on teeth, oral dry, gingival retraction, carious) (Fig. 3).

## 3. Change in gut microbiota by oral condition

The top 30 gut microorganisms are shown in Fig. 4. The highest relative abundance microorganisms were *Bacteroides*, *Feecalibacterium*, and *Blautia*. We compared the relative abundance of gut microorganisms according to oral conditions. As shown in Fig. 5, Spearman's correlation coefficients between negative and positive group were showed high correlation (all correlation coefficients  $\rho > 0.85$ ). So the gut microbiomes displayed a similar structure according to oral conditions.

However, several important gut microorganisms

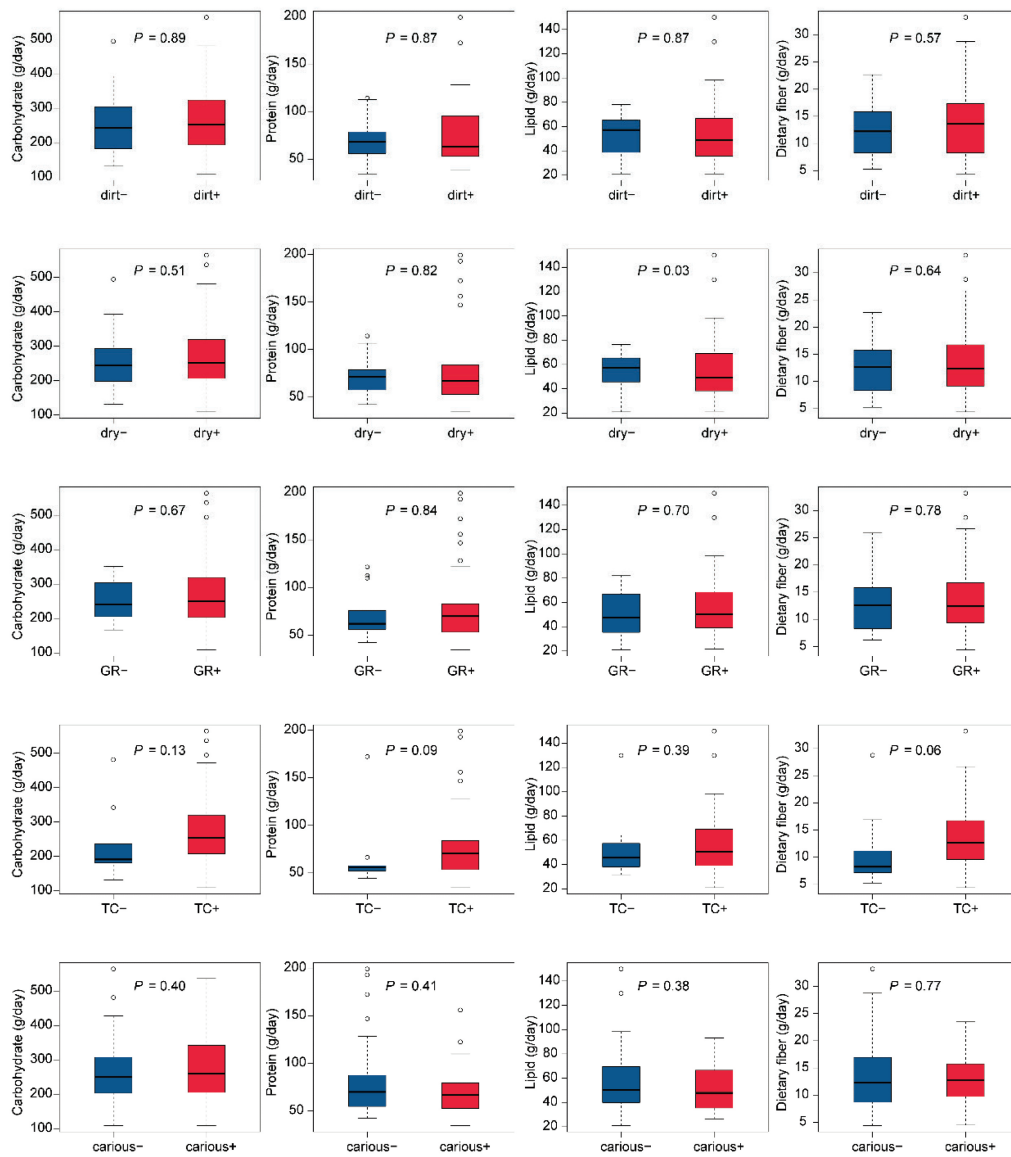


Figure 3. Comparison of nutrient intake by oral conditions. The following oral conditions were considered: dirt on teeth (dirt), oral dry (dry), gingival recession (GR), tongue coating (TC), and carious. There were no significant differences according to the Mann-Whitney U test.

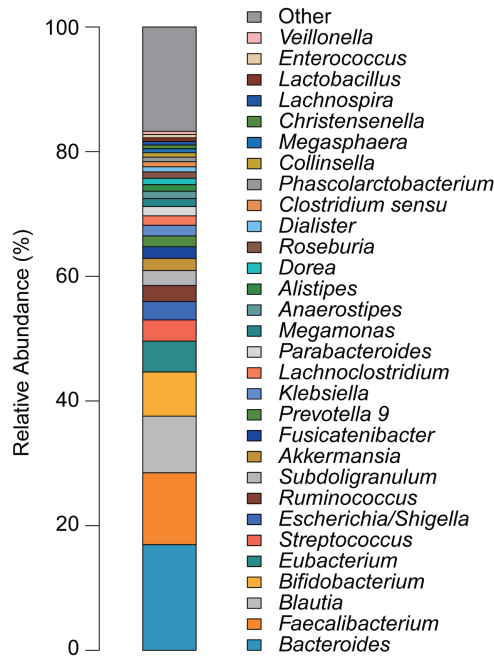


Figure 4. Structure of gut microbiome of all participants. The bar graph shows the relative abundance of the top 30 microorganisms.

were altered by oral conditions (Fig. 6). *Escherichia/Shigella* was increased with denture use and decreased in dry oral conditions (denture use:  $P = 0.04$ , oral dry:  $P = 0.01$ ) (Fig. 6A). *Klebsiella* was increased by dirt on teeth and dry oral conditions (dirt on teeth:  $P < 0.01$ , oral dry:  $P = 0.04$ ) but was decreased by gingival recession ( $P < 0.01$ ) (Fig. 6B). *Lactobacillus* was decreased by gingival recession and carious (gingival recession:  $P < 0.01$ , carious:  $P = 0.04$ ) (Fig. 6C).

#### 4. Correlation of gut microorganisms and nutrient intake

We examined the relationships between gut microorganisms and nutrient intakes. Table 2 shows the Spearman's correlation coefficients according to the relative abundance of microorganisms and nutrient intake. There was a very weak negative correlation between dietary fiber intake and the relative abundance of *Escherichia/Shigella* ( $\rho = -0.20$ ,  $P = 0.03$ ) (Table 2). Several other microorganisms had

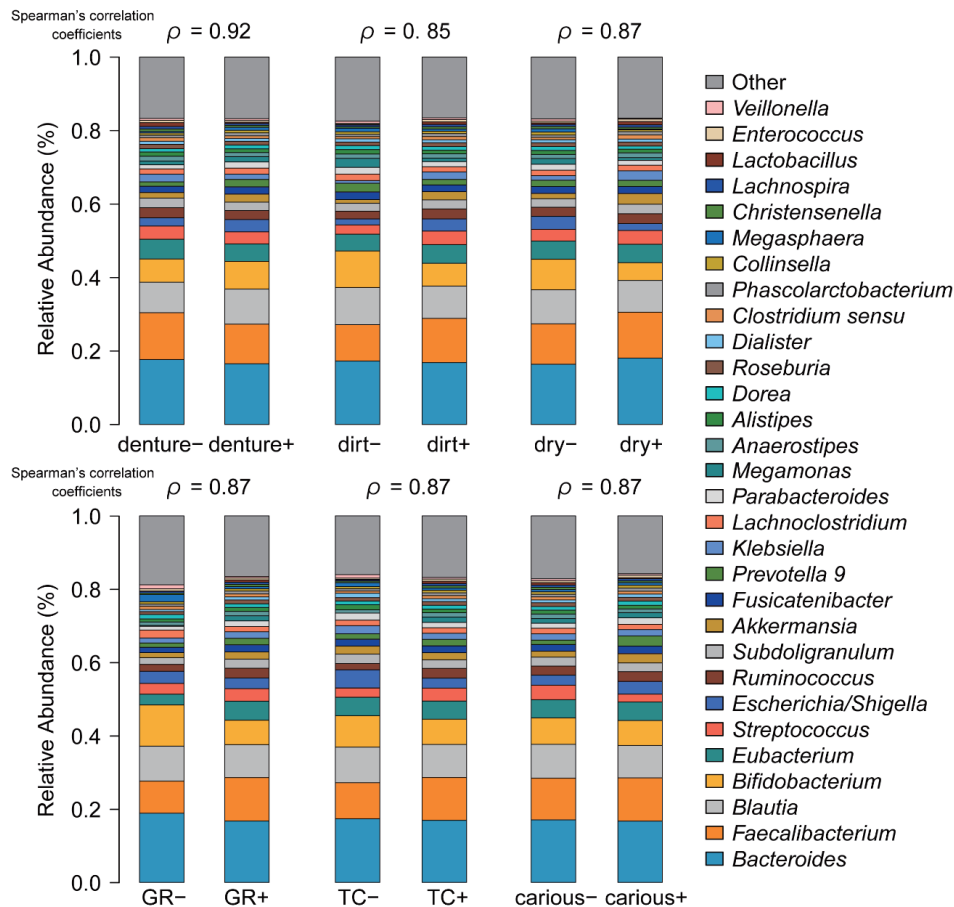


Figure 5. Comparison of microorganism relative abundance by oral conditions. Relative abundance of the top 30 microorganisms. The following oral conditions were considered: denture use (denture), dirt on teeth (dirt), oral dry (dry), gingival recession (GR), tongue coating (TC), and carious. Spearman's correlation coefficients ( $\rho$ ) were used to determine the similarity of the microbiome between each oral condition- and +.



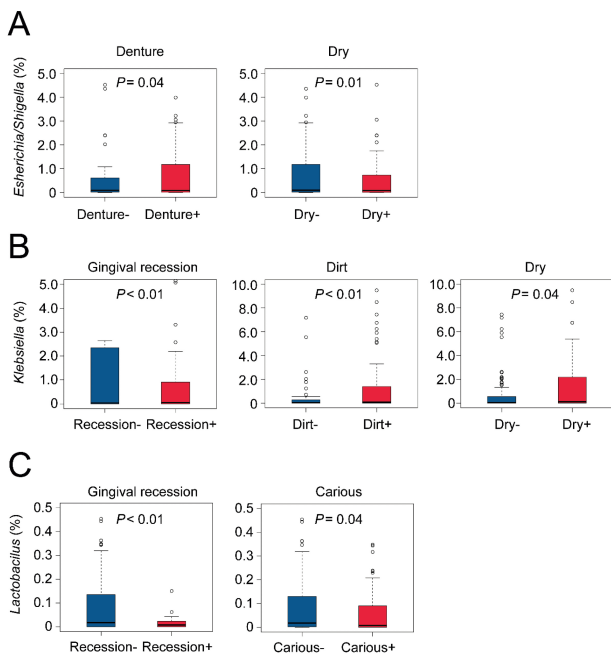


Figure 6. Comparison of bacterial relative abundance by oral conditions. The box plot shows microorganisms that were significantly different among the groups. The following oral conditions were considered: denture use (Denture), dirt on teeth (Dirt), oral dry (Dry), gingival recession, and carious. (A) *Escherichia/Shigella* was increased with denture use and decreased with dry oral conditions. (B) *Klebsiella* was increased by dirt on teeth and dry oral conditions and was decreased by gingival recession (GR). (C) *Lactobacillus* was decreased by GR and carious. DESeq2 was used as the significance test.

very weak correlations with nutrient intake. There were no correlations between the relative abundance of *Klebsiella* and *Lactobacillus* and nutrient intake (Table 2).

## Discussion

We examined the relationship between oral condition, nutrient intake, and the gut microbiome and found that denture use altered nutrient intake. Specifically, denture use increased the relative abundance of *Escherichia/Shigella*. *Escherichia/Shigella* had a weak negative correlation with dietary fiber intake. *Klebsiella* and *Lactobacillus* were also affected by several oral conditions. In this research, Participants were 40 years of age or more. These people start loss of teeth and using denture. These participants are living in Shika town Ishikawa prefecture in Japan. Shika town is located in a rural area, not in a city area. The collected subjects in this study may not be the general residents in Japan. And we cannot examine participants' basic diseases, so participants' health condition were not

uniform.

Denture use was related to nutrient intake after adjustments for age. Reducing the number of teeth causes inadequate occlusion and decreases masticatory forces. In the present study, dietary fiber intake was decreased among denture-wearers. Previous studies demonstrated that the intake of vegetables (rich in dietary fiber) among denture-wearers was 1.5 times less than that of the fully dentate<sup>27</sup>; thus, fiber intake was 1.2 times lower among denture-wearers than among the fully dentate<sup>27</sup>. Furthermore, denture use also significantly reduced protein intake. Yong *et al.* showed that after adjusting for sociodemographic characteristics, protein intake was positively associated with the total number of natural teeth<sup>28</sup>. These studies suggest that decreasing the remaining teeth or using dentures causes oral feeding difficulties with vegetables or foods high in protein; thus, changes in health conditions caused by changes in nutrition may be related to denture use.

In this study, nutrient intake, particularly of dietary fiber, was affected by many gut microorganisms<sup>29-30</sup>. Dietary fiber is the most common fuel for gut microbiota. A single human gut microbiota may contain upwards of 60,000 carbohydrate-degrading enzymes<sup>31</sup>. Dietary fiber is metabolized to short-chain fatty acids (SCFAs, including butyrate, propionate, and acetate) by these enzymes. SCFAs play many important roles affecting health conditions in the host. Low dietary fiber intake has been reported to reduce the abundance of several micrograms and decrease beneficial microbial metabolites such as SCFAs<sup>8, 29</sup>. Additionally, the colonic microbial digestion of protein generates various end products including SCFAs<sup>7, 10</sup>. In this study, the denture-wearer group, which had a lower intake of dietary fiber and protein, had an increased abundance of *Escherichia/Shigella*. High dietary fiber diets have been reported to decrease the relative abundance of *Escherichia*<sup>12, 32, 33</sup>. Therefore, we suggested that denture use causes a low dietary fiber intake, resulting in increasing *Escherichia/Shigella* in the gut.

*Escherichia/Shigella* and *Klebsiella* increase not only with denture use but also in the presence of dirt on teeth and dry oral conditions. These microorganisms have been reported to cause several status of inflammation in the colon<sup>34</sup>, and patients with inflammatory bowel disease have a high relative

Table 2. Relationship of nutrient intake and relative abundance of each microorganism. Spearman's correlation coefficients were calculated. The correlation coefficients showed all combinations of nutrient intakes and the relative abundance of each microorganism. Significant correlation coefficients are marked with asterisks. \* $P < 0.05$ , \*\* $P < 0.01$ .

Gut micrograms	Spearman's correlation coefficient Gut micrograms vs nutrients intake			
	Carbohydrate	Protein	Lipid	Dietary fiber
<i>Bacteroides</i>	0.09	0.09	-0.01	0.14
<i>Faecalibacterium</i>	0.07	0.07	-0.06	0.17
<i>Blautia</i>	0.06	0.06	0.1	0.11
<i>Bifidobacterium</i>	-0.09	-0.04	-0.06	-0.15
<i>Eubacterium</i>	0.1	0.09	-0.02	0.15
<i>Streptococcus</i>	0.18	0.14	0.17	0.15
<i>Escherichia/Shigella</i>	0.18	0.04	-0.03	<b>-0.20*</b>
<i>Ruminococcus</i>	0.12	0.11	0.02	<b>0.26**</b>
<i>Subdoligranulum</i>	0.05	0.07	-0.04	0.15
<i>Akkermansia</i>	-0.04	0.07	-0.07	0.03
<i>Fusicatenibacter</i>	-0.05	-0.05	-0.08	0
<i>Prevotella 9</i>	0.02	-0.06	-0.09	-0.05
<i>Klebsiella</i>	0.11	0.04	0.14	0.05
<i>Lachnoclostridium</i>	0.04	0.11	0.01	0.05
<i>Parabacteroides</i>	-0.03	0.01	-0.09	-0.03
<i>Megamonas</i>	-0.08	-0.1	-0.08	-0.04
<i>Anaerostipes</i>	-0.08	-0.08	-0.05	0.02
<i>Alistipes</i>	0.05	0.07	-0.08	0.13
<i>Dorea</i>	0.04	0.03	0.08	-0.04
<i>Roseburia</i>	<b>0.21*</b>	0.17	0.02	<b>0.18*</b>
<i>Dialister</i>	-0.18	-0.17	<b>-0.23*</b>	-0.11
<i>Clostridium sensu</i>	0.1	0.11	-0.04	0.06
<i>Phascolarctobacterium</i>	0.1	0.16	0.07	0.08
<i>Collinsella</i>	-0.03	-0.08	0.01	-0.08
<i>Megasphaera</i>	0.05	0.08	0.15	-0.07
<i>Christensenella</i>	-0.12	-0.08	<b>-0.21*</b>	0.05
<i>Lachnospira</i>	0.04	0.08	-0.03	0.12
<i>Lactobacillus</i>	0.11	0.11	0.07	0.17
<i>Enterococcus</i>	0.09	0.06	0.17	0.05
<i>Veillonella</i>	0.03	0.05	-0.12	-0.12

Each value was Spearman's correlation coefficient between each micrograms and nutrient intake. Significant correlation coefficients were marked with asterisks. \* $P < 0.05$ , \*\* $P < 0.01$

abundance of *Escherichia* and *Klebsiella*<sup>9, 35</sup>). These microorganisms are gram-negative bacteria that produce LPS. LPS purified from *E. coli* has also led to anxiety and colitis in mice<sup>9, 34</sup>). In this study, denture use, dirt on teeth, and dry oral conditions increased the abundance of *Escherichia* and *Klebsiella*. This result suggests that poor oral conditions and denture use may cause the onset of several diseases.

The limitation of this study, since this study was cross-sectional, the participants were not investigated over time. Thus, it remains elusive whether deteriorating oral conditions and the loss of teeth induces the growth of *Escherichia/Shigella* and *Klebsiella* over time in the

intestine due to reduced dietary fiber and protein intake. We also did not perform a metabolomics analysis; thus, we do not know what type of metabolome is formed in the intestine due to the increase of *Escherichia/Shigella* and *Klebsiella* and the nutritional condition is biased, and what type of change causes to the health condition. We cannot measure whole body nutrition index such serum albumin. But BMI was not changed by oral condition.

Consequently, we examined the relationship between oral condition, nutrient intake, and the gut microbiome. The results suggest that changing oral conditions may lead to the deterioration of general conditions.

This research has many unclear findings that should be clarified and require further study in the future. However, our present study is very significant because we suggest that the oral condition, which no one had previously excluded, could be a factor affecting the intestinal environment and health. In the future, if further research determines that the oral condition does in fact affect the intestinal environment and health, the importance of oral care for health maintenance and disease prevention may be clarified with scientific evidence.

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## 石川県における健常成人の口腔状態と腸内細菌叢の関連

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### 要 旨

口腔状態は、食習慣や栄養摂取に影響を及ぼす可能性が考えられ、栄養摂取量によって腸内細菌叢の構成は変化する可能性も考えられる。また、腸内細菌叢は宿主の健康状態と強く関連することも推察されている。しかし、ヒトにおいてその可能性について包括的に検討した報告は殆ど存在しない。本研究において、我々は石川県羽咋郡志賀町に住む一般住人 239 名を対象に、対象者の口腔状態、栄養摂取量、腸内細菌叢を調査し、これらの関連性について検討した。その結果、入れ歯の使用者は食物繊維の摂取量が有意に少なく、さらに腸内細菌の *Escherichia/Shigella* の存在比率が有意に高値であることを明らかにした。また、いくつかの口腔状態の差異によって複数の腸内細菌叢の存在比率に差異を認めた。高い食物繊維の摂取が腸内の *Escherichia/Shigella* の存在比率を低下させるとの報告が存在することから、入れ歯の着用により食物繊維の摂取量が減少し、これが腸内の *Escherichia/Shigella* の存在比率上昇の一因である可能性が示唆された。*Escherichia/Shigella* は腸内の炎症を引き起こすなど、本研究では入れ歯の使用などの口腔状態の変化がこのような疾患を引き起こす可能性を示唆した。