INTRODUCTION

Toothbrushes are generally used to remove dental plaque and some studies demonstrate the presence of bacteria on toothbrushes [1-3]. Many bacteria are found on toothbrushes after brushing [4], and these bacteria can survive for one day to one week after brushing [5]. Toothbrushes can be contaminated by the oral environment, hands, and contaminated containers [6]. Contaminated toothbrushes can cause oral disease, sepsis, and many systemic diseases of the cardiovascular system, respiratory organs, and the kidneys [7].

Various studies reported the number and types of microorganisms present on toothbrushes and reported contamination by pathogenic microorganisms after using toothbrushes [8-13]. An average of $10^3$ to $10^5$ colony-forming unit (CFU) of bacteria were found per toothbrush. Malmberg et al. [10] examined the distribution of microorganisms in 44
toothbrushes collected from four nurseries. Streptococci like *Streptococcus salivarius*, *Streptococcus sanguinis*, and *Streptococcus mitis* were most prevalent in these studies, and *Haemophilus* was found in 82% of the samples. Researchers also found oral microorganisms associated with periodontal disease and fungi. Another study found that 70% of toothbrushes were contaminated with pathogenic microorganisms after use [14].

Most of these studies used a method of culturing bacteria in a specific medium. The identified microorganisms were identified using Gram staining and biochemical characteristics. However, this identification method is limited because it cannot detect microorganism species that are not cultured. Sometimes, identification using biochemical properties may not match the characteristics of any known species or genus.

To overcome these limitations, this study used Illumina sequencing to identify microorganisms present on toothbrushes.

**MATERIALS AND METHODS**

**Sampling**

A total of 10 toothbrush samples were obtained by collecting 2 toothbrushes (group A: toothbrushes stored in the office, group B: toothbrushes stored in the bathroom) from 5 healthy adults (aged 20–50). The toothbrush sample was transferred to a test tube containing 10 mL of sterile distilled water and vortexed for 5 minutes to remove the bacteria from the head of toothbrush.

**Genomic DNA extraction**

The PureLink® Genomic DNA Mini Kit (Invitrogen, Waltham, MA, USA) was used to extract DNA from the 10 toothbrushes and DNA was extracted according to the manufacturer’s instructions. For Illumina sequencing, individual DNA samples belonging to each group were mixed in equal proportions.

**RESULTS**

**Bacterial diversity of toothbrush samples**

Table 1 shows the microbial diversity of the two toothbrush groups with different storage locations (group A, group B). In group A, 78,171 reads and 126 genera were obtained, while 83,021 reads and 151 genera were obtained in group B. In group A, *Enterococcus* (30.76%), *Pseudomonas* (21.85%), *Streptococcus* (14.94%) and *Lactobacillus* (5.15%) were most abundant. In group B, *Strep-
Table 1. List of genera identified on toothbrushes using Illumina MiSeq

| Phylum          | Class      | Genus                | Count | Count |
|-----------------|------------|----------------------|-------|-------|
|                 |            |                      | Group A | Group B |
| **Bacteria**    |            |                      |       |       |
| Actinobacteria  | Actinobacteria_c | Actinobaculum         | 13    | 7     |
|                 |            | Actinomyces          | 401   | 786   |
|                 |            | Antricoccus          | -     | 11    |
|                 |            | Arsenicococcus       | -     | 11    |
|                 |            | Bifidobacterium      | 282   | 403   |
|                 |            | Brachybacterium      | -     | 15    |
|                 |            | Brevibacterium       | 131   | 218   |
|                 |            | Corynebacterium      | 69    | 99    |
|                 |            | Dietzia              | -     | 4     |
|                 |            | Gardnerella          | -     | 7     |
|                 |            | Kocuria              | 32    | 583   |
|                 |            | Microbacterium       | 3     | -     |
|                 |            | Phycicoccus          | -     | 2     |
|                 |            | Propionibacterium    | 4     | 6     |
|                 |            | Rhodococcus          | 3     | 70    |
|                 |            | Rothia               | 1,557 | 2,908 |
|                 |            | Atopobium            | 29    | 130   |
|                 |            | Collinsella          | 9     | 14    |
|                 |            | Senegalimassilia     | -     | 8     |
| Coriobacteriia  |            |                      |       |       |
|                  |            | Alistipes            | 58    | 890   |
|                  |            | Alloprevotella       | 125   | 959   |
|                  |            | Bacteroides          | -     | 221   |
|                  |            | Dysgonomonas         | 2     | -     |
|                  |            | Marinilimophilum     | 1     | -     |
|                  |            | Parabacteroides      | -     | 20    |
|                  |            | Porphyromonas        | 152   | 937   |
|                  |            | Prevotella           | 861   | 3,813 |
|                  |            | Tannerella           | 3     | 72    |
|                  |            | Bergeyella           | 17    | 88    |
|                  |            | Capnocytophaga       | 81    | 162   |
|                  |            | Chryseobacterium     | -     | 14    |
|                  |            | Maritimimonas        | -     | 2     |
|                  |            | Sphingobacterium     | 5     | 140   |
| Flavobacteria    |            |                      |       |       |
|                  |            | Atopobium            | 29    | 130   |
|                  |            | Collinsella          | 9     | 14    |
|                  |            | Senegalimassilia     | -     | 8     |
|                  |            | Alistipes            | 58    | 890   |
|                  |            | Alloprevotella       | 125   | 959   |
|                  |            | Bacteroides          | -     | 221   |
|                  |            | Dysgonomonas         | 2     | -     |
|                  |            | Marinilimophilum     | 1     | -     |
|                  |            | Parabacteroides      | -     | 20    |
|                  |            | Porphyromonas        | 152   | 937   |
|                  |            | Prevotella           | 861   | 3,813 |
|                  |            | Tannerella           | 3     | 72    |
|                  |            | Bergeyella           | 17    | 88    |
|                  |            | Capnocytophaga       | 81    | 162   |
|                  |            | Chryseobacterium     | -     | 14    |
|                  |            | Maritimimonas        | -     | 2     |
|                  |            | Sphingobacterium     | 5     | 140   |
|                  |            | Aerococcus           | -     | 1     |
|                  |            | Alkalibacterium      | 1     | -     |
|                  |            | Bacillus             | 55    | -     |
|                  |            | Enterococcus         | 24,088| 6,790 |
|                  |            | Gemella              | 232   | 1,994 |
|                  |            | Granulicatella       | 205   | 309   |
|                  |            | Lactobacillus        | 4,032 | 647   |
|                  |            | Lactococcus          | 98    | 3     |
|                  |            | Staphylococcus       | 64    | -     |
|                  |            | Streptococcus        | 11,703| 16,410|
|                  |            | Vagococcus           | 1     | -     |
|                  |            | Weissella            | 7     | -     |
| **Clostridia**  |            |                      |       |       |
|                  |            | Acetatitfactor       | -     | 7     |
|                  |            | Acidaminobacter      | 81    | 3     |
|                  |            | Agathobacter         | -     | 4     |
|                  |            | Alkalibacter         | 37    | -     |

**Note:** The table includes counts of genera identified on toothbrushes using Illumina MiSeq technology.
Table 1. Continued

| Phylum         | Class          | Genus           | Count | Group A | Group B |
|----------------|----------------|-----------------|-------|---------|---------|
|                |                | Alkaliphilus    | 31    |         |         |
|                |                | Anaerovorax     | 3     |         |         |
|                |                | Blautia         | 5     |         | 32      |
|                |                | Butyricoccus    | -     |         | 5       |
|                |                | Caproicproduens | -     |         | 2       |
|                |                | Catonella       | 8     |         | 70      |
|                |                | Clostridium     | 12    |         | 129     |
|                |                | Dorea           | 7     |         | 13      |
|                |                | Eisenbergiella  | 23    |         | 597     |
|                |                | Eubacterium     | 42    |         | 210     |
|                |                | Faecalibacterium| 6     |         | 64      |
|                |                | Filifactor      | -     |         | 4       |
|                |                | Fusicatenibacter| 6     |         | 10      |
|                |                | Intestinibacter | 4     |         | -       |
|                |                | Lachnoanaerobaculum| 45   |         | 74      |
|                |                | Lachnospira     | -     |         | 14      |
|                |                | Marvinbryantia  | -     |         | 1       |
|                |                | Mogibacterium   | 1     |         | -       |
|                |                | Morrellia       | 45    |         | 69      |
|                |                | Oribacterium    | 57    |         | 73      |
|                |                | Oscillibacter   | 5     |         | 107     |
|                |                | Peptostreptococcus| 25  |         | 190     |
|                |                | Pseudoflavonifractor| 3   |         | 126     |
|                |                | Romboutsia      | 3     |         | 5       |
|                |                | Roseburia       | -     |         | 6       |
|                |                | Ruminococcus    | 14    |         | 31      |
|                |                | Sporobacter      | -     |         | 5       |
|                |                | Subdoligranulum | -     |         | 11      |
|                |                | Tindallia       | 11    |         | -       |
|                | Erysipelotrichi | Bulleidia       | 32    |         | 36      |
|                |                | Catenibacterium | -     |         | 14      |
|                |                | Holdemanella    | 2     |         | 6       |
|                | Negativicutes   | Dialister       | 2     |         | 12      |
|                |                | Megamonas       | -     |         | 5       |
|                |                | Megasphaera     | 5     |         | 14      |
|                |                | Pelosinus       | 10    |         | 10      |
|                |                | Phascolarctobacterium| -  |         | 9       |
|                |                | Selenomonas     | 21    |         | 137     |
|                |                | Veillonella     | 1,261 |         | 3,757   |
|                | Tissierellia    | Dethiosulatibacter| 114 |         | -       |
|                |                | Parvimonas      | 24    |         | 141     |
|                |                | Tissierella     | 157   |         | -       |
|                | Fusobacteria    | Fusobacterium   | 400   |         | 1,923   |
|                |                | Leptotrichia    | 111   |         | 893     |
|                |                | Streptobacillus | -     |         | 16      |
|                | Proteobacteria  | Alphaproteobacteria| 467 |         | 18      |
|                |                | Altererythrobacter| -   |         | 7       |
|                |                | Auremonas       | 13    |         | 15      |
|                |                | Brevundimonas   | 56    |         | 7       |
|                |                | Novosphingobium | 5     |         | -       |
|                |                | Rhizobium       | 467   |         | 18      |
|                |                | Rhodobacter     | -     |         | 5       |
### Table 1. Continued

| Phylum            | Class          | Genus          | Count | Group A | Group B |
|-------------------|----------------|----------------|-------|---------|---------|
|                   |                |                |       |         |         |
| Betaproteobacteria|                |                |       |         |         |
|                   |                | Roseomonas     | 18    | 2       |         |
|                   |                | Sphingobium    | 8     | 5       |         |
|                   |                | Sphingomonas   | 17    | 2       |         |
|                   |                | Acidovorax     | 13    | 13      |         |
|                   |                | Comamonas      | 3     | 7       |         |
|                   |                | Curvibacter    | 6     | 6       |         |
|                   |                | Dechloromonas  | 29    | 18      |         |
|                   |                | Delfta         | 61    | 35      |         |
|                   |                | Duganella      | 3     | -       |         |
|                   |                | Eikenella      | -     | 1       |         |
|                   |                | Herbaspirillum | 3     | -       |         |
|                   |                | Janthinobacterium | 3,393 | 2,110   |         |
|                   |                | Kingella       | 11    | 65      |         |
|                   |                | Lautropia      | 23    | 198     |         |
|                   |                | Neisseria      | 1,067 | 5,854   |         |
|                   |                | Paucibacter    | 53    | 43      |         |
|                   |                | Simonsiella    | -     | 7       |         |
|                   |                | Sutterella     | -     | 4       |         |
|                   |                | Thauera        | 2     | 1       |         |
|                   |                | Undibacterium  | 6     | 11      |         |
| Deltaproteobacteria| Desulfonatronum| 194            | 10    |         |         |
| Epsilonproteobacteria| Arcobacter    | 15              | 114   |         |         |
|                   | Campylobacter  | 15              | 114   |         |         |
|                   | Helicobacter   | -               | 1     |         |         |
|                   | Sulfurovum     | 2               | 3     |         |         |
| Gammaproteobacteria| Actinobacter   | 2,937           | 840   |         |         |
|                   | Actinobacillus | 3               | 101   |         |         |
|                   | Aeromonas      | 389             | 264   |         |         |
|                   | Aggregatibacter| 7               | 122   |         |         |
|                   | Buttiauxella   | 10              | 18    |         |         |
|                   | Cardiobacterium| 12              | 37    |         |         |
|                   | Cedecea        | 68              | 36    |         |         |
|                   | Citrobacter    | 27              | 12    |         |         |
|                   | Enhydrobacter  | 195             | 332   |         |         |
|                   | Enterobacter   | 458             | 129   |         |         |
|                   | Erwinia        | 1,707           | 173   |         |         |
|                   | Escherichia    | 43              | 11    |         |         |
|                   | Haemophilus    | 556             | 2,047 |         |         |
|                   | Halomonas      | 23              | 16    |         |         |
|                   | Halotalea      | 3               | -     |         |         |
|                   | Klebsiella     | 5               | 156   |         |         |
|                   | Moraxella      | -               | 3     |         |         |
|                   | Morganella     | 143             | 132   |         |         |
|                   | Pantoea        | 133             | 4,109 |         |         |
|                   | Pseudoalteromonas | -              | 1     |         |         |
|                   | Pseudomonas    | 17,110          | 13,374|         |         |
|                   | Rahmella       | 15              | 5     |         |         |
|                   | Serratia       | -               | 8     |         |         |
|                   | Stenotrophomonas| 1,081           | 761   |         |         |
|                   | Vibrio         | 2               | 8     |         |         |
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tococcus (19.73%), Pseudomonas (16.08%), Enterococcus (8.16%) and Neisseria (7.04%) were most abundant (Fig. 2–4).

**Pathogenic bacteria**

In group A, 45 out of 124 genera (36.29%) belonged to pathogenic species. *Enterococcus* was the most common genus, and we also found pathogenic species in the genera *Pseudomonas*, *Acinetobacter*, *Neisseria*, *Haemophilus*, *Corynebacterium*, *Staphylococcus*, *Bacillus*, and *Escherichia*. We also detected bacteria associated with oral diseases like *Streptococcus*, *Actinomyces*, *Fusobacterium*, *Porphyromonas*, *Prevotella*, *Pseudomonas*, *Rothia*, *Streptococcus*, *Veillonella*, and *Etc. (<1%)*

**DISCUSSION**

The toothbrush is contaminated from the point of use, and the contamination increases when the toothbrush is used repeatedly [18,19]. In addition, 80% of toothbrushes are known to be contaminated before use [8]. The number of bacteria found on each toothbrush is highly variable and was reported to average from 10^3 to 10^5 CFU per toothbrush [8,10,12,13]. Glass and Lare [19] examined the microbial distribution on toothbrushes used by adults and found various genera, including pathogenic species like *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas*.

In addition, Malmberg et al. [10] investigated children’s toothbrushes, finding mostly aerobic microorganisms and bacteria like *Staphylococci* and *Pseudomonas*, and fungi. In a study by Mehta et al. [14], 70% of toothbrushes used in the experiment were found to be severely contaminated by pathogenic microorganisms. Studies on microbial contamination of toothbrushes have been done before, but most
of them have identified bacteria based on general bacterial culture methods. Therefore, the risk of contaminated toothbrushes may be underestimated.

In our study, the Enterococcus genus known to have multi-drug resistance was found most frequently. We also found the genus Pseudomonas, an opportunistic infectious bacteria, and Streptococcus, a bacterium related to oral diseases. These results were similar to most other studies, but there were differences in the abundances found for each group. Among the toothbrushes stored in office, Enterococcus was observed at the highest abundance, followed by Pseudomonas, Streptococcus, and Lactobacillus. The Streptococcus genus was most frequently observed in toothbrushes stored in the bathroom, followed by Pseudomonas, Enterococcus, and Nisseria. The proportion of genera containing pathogenic species was observed to be 36.29% in group A and 33.77% in group B. In both groups, we found Haemophilus, which can cause a deadly respiratory infection, Corynebacterium, which is known to be the causative agent of diphtheria, and Neisseria, which can cause septicemia and gonorrhea. In group A, we found Bacillus and Staphylococcus. In group B, we found Helicobacter, which can cause stomach inflammation, gastric cancer, and peptic ulcer disease. In addition, we found several oral bacteria that are related to oral diseases. In both groups, we also found Streptococcus and Actinomyces, causative bacteria of dental caries, and Porphyromonas, Fusobacterium, Aggregatibacter, Tannerella, and Treponema, which are associated with periodontal disease.

Repeated use of contaminated toothbrushes may increase the possibility of bacterial infection [18]. We also found pathogenic bacteria that do not directly cause infection in healthy individuals but could be fatal to patients with weak immune systems. Thus, there is a need to reduce microbial

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Fig. 3. Bacterial community diversity in toothbrush samples (group A, toothbrushes stored in the office).
contamination of toothbrushes. The contamination of the toothbrush can be influenced by factors like the shape of the toothbrush, the toothpaste, the storage of the toothbrush, the frequency of brushing, and the period of use. We obtained toothbrush samples in two groups according to storage location (group A, group B). Because toothbrushes are often stored in bathrooms, they can be exposed to enteric bacteria that are aerosolized [12]. We expected enteric bacteria to be found only in toothbrush samples stored in the bathroom, but enteric bacteria were isolated in both groups of toothbrushes. The toothbrush stored in the office seems to be contaminated with enteric bacteria via the human hand or other infection routes.

Based on previous studies, patients with systemic diseases are advised to frequently replace their toothbrush [4,9,20]. The American Dental Association recommends replacing the toothbrush every three months [4], and patients with oral disease also reported reduced symptoms after replacing toothbrushes [9]. Many previous studies demonstrated that brushing is effective in improving oral hygiene [21,22]. There is also a risk of pathogenic microbial transfer due to toothbrush contamination and the positive role of such a toothbrush. Therefore, further research is needed to propose a method to reduce toothbrush contamination and establish better hygiene standards.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

Fig. 4. Bacterial community diversity in toothbrush samples (group B, toothbrushes stored in the bathroom).
Bacterial contamination of toothbrushes

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