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Proposal for salivary auto-transplantation as a probiotic against radiation-induced stomatitis

Masaru Ohara[1], Ikue Hayashi[2], Hiroaki Takakura[3], Daisuke Yamamoto[3], Hiroshi Mese[3]

[1] *School of Dental Hygiene, Ogaki Women's College, Ogaki, Japan.*
[2] *Central Research Facility, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan.*
[3] *Department of Dentistry and Oral Surgery, Fukuyama City Hospital, Fukuyama, Japan*
Corresponding author: Masaru Ohara DDS, PhD. School of Dental Hygiene, Ogaki Women's College, 1-109 Nishinokawa Ogaki city Gifu prefecture, 503-8554, Japan.

ABSTRACT: *Radiation therapy (RT) is an effective treatment for head and neck cancer. However, patients suffer from radiation-induced oral mucositis (OM) and/or xerostomia as a side-effect of therapy. Studies have indicated that oral dysbiosis is associated with the exacerbation of RT-OM. In this study, we evaluated how RT was performed on patients in a hospital. In Japan, systemic antibiotics are administered in a few cases for prophylaxis of oral infection, which raises questions on the necessity of the practice. However, physicians are required to reduce or prevent patient symptoms at the bedside. We evaluated three microbiome datasets that showed dysbiosis during real clinical treatments and devised a new method to reduce RT-OM using the patient's oral flora in three steps: (i) collecting saliva before RT, (ii) storing the collected saliva by freezing, (iii) auto-transplanting the stored saliva after RT-OM onset. Unfortunately, we have not yet found collaborators to perform this method. Therefore, we attempted to study whether collected oral flora can be cultured and amplified without changing the microbial ratios. We discuss the potential of using saliva as a probiotic. In the future, artificial control of oral flora may prevent some oral diseases by re-establishing a healthy oral ecosystem.*

Keywords: *microbiome, oral flora, dysbiosis, probiotics*

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I. INTRODUCTION

The oral cavity contains 600–700 species of microorganisms which are referred to as oral flora [1]. At birth, the oral ecosystem is influenced by the oral bacteria derived from the mother (caregiver). The digestive tract, vagina, skin, breast milk, and baby food are sources of microorganisms from mothers or caregivers [2]. After initial colonization, the human microbiome is significantly influenced by environmental exposure [3]. Physical, chemical, and biological conditions enable the creation of micro-ecosystems via colonization by environmentally derived bacteria [4]. From the neonatal period to adulthood, the oral microbiome becomes significantly more diverse and mature according to the active lifestyles followed [5]. In addition to physical, chemical, and biological factors influencing the oral environment, geography, ethnicity, and lifestyle-specific variations, including differences in host genetics and innate/adaptive immunity may also have an impact on the oral microbiome composition [6].

Next-generation sequencing (NGS) technology has enabled the analysis of a large amount of data on the oral microbiome [7-10]. Using NGS, oral dysbiosis in the oral microbiome associated with oral diseases such as dental caries, periodontal disease, and oral cancer has been studied [11-19]. Additionally, oral dysbiosis is also associated with systemic diseases such as diabetes, autoimmune, and cardiovascular diseases [20-22]. Accumulating data on the oral microbiome must be used to understand the pathogenesis and etiology of oral lesions and related systemic diseases. However, the focus of our study is slightly different; from a clinical viewpoint, we analyzed how controlling the oral ecosystem can cure or prevent oral diseases. We evaluated how radiation therapy (RT) for patients with head and neck (HN) cancer induces oral microbiome changes after adulthood because of therapeutic side effects.

RT is a therapeutic tool for treating head and neck (HN) cancer [23]. Recently, intensity-modulated radiation therapy (IMRT) and several other techniques have yielded highly conformal dose distributions [24], resulting in toxicity reduction and faster treatment times. However, the associated adverse effects range from oral mucositis, mucosal opportunistic infections, neurosensory disorders, and tissue fibrosis [25]. Radiation therapy also induces salivary gland hypofunction, resulting in a change in saliva and oral dryness, termed

xerostomia [23, 26]. The microbiological changes after head and neck radiation therapy (HN-RT) have been reviewed in many papers [27-32].

On the other hands, the relationship between dysbiosis and oral mucositis during RT for head and neck cancer was summarized in a few studies (Table 1). Ingrosso et al. [33] showed that the oral microbiome diversity decreases during treatment phases II and III, which corresponded to ~2–3 weeks of HN-RT. Zhu et al. [34] showed that bacterial community structure was progressively altered during RT, in parallel with a marked increase in the relative abundance of a few types of Gram-negative bacteria. Hu et al. [35] found that the microbial composition of oral plaques changes during HN-RT. A negative correlation between the number of operational taxonomic units (OTUs) and radiation dose was observed. Vesty et al. [36] reported that the salivary microbiome remained stable during RT, but buccal mucosa swab samples showed an abundance of several Gram-negative bacilli (GNB). The group also found that the salivary microbiota is consistently dominated by the genera *Streptococcus*, *Prevotella*, *Fusobacterium*, and *Granulicatella*. Obligate and facultative anaerobic GNB Bacteroidales G2, *Capnocytophaga*, *Eikenella*, *Mycoplasma*, and *Sneathia*, as well as anaerobic GNB in the periopathogenic genera *Porphyromonas* and *Tannerella*, were all positively correlated with ≥ grade 2 OM.

Although sampling procedures and factors such as place or timing affect microbiome data, oral dysbiosis is strongly related to RT-OM. In real-life clinical situations, physicians administer extra medications such as steroids, antibiotics, and anti-fungal medications to patients with RT-OM, and these medications are likely to affect the oral microbiome. At the beginning of this study, we observed changes in the microbiomes of three patients with HN-RT. Two of the three patients had been administered antibiotics and/or other drugs.

Among the factors that exacerbate oral mucositis, improvement of dysbiosis is one of the treatments for RT-induced adverse effects. We hypothesized that collecting saliva before radiation therapy can be used as a probiotic after the onset of RT-induced stomatitis. Administration of the collected oral flora induced a return to the oral environment that was prevalent before RT. To perform this study, we drew a blueprint consisting of three steps, as shown in Fig 2 (discussed in detail later). In the first step, saliva was collected from a patient before radiation therapy. Second, the collected saliva was stored in a freezer. Although the third step is the most important part of the study, it requires the cooperation of participants and hospitals to perform clinical challenges. In this study, we focused on the second step of the blueprint. We aimed to culture/amplify oral microbiota to use it for auto-transplantation after the onset of RT-OM, and discuss methods for controlling the oral flora to prevent oral diseases. We would like to outline our hypothesis to readers and discuss the feasibility of the approach.

Table 1. summary of oral microbiome analysis during RT

oral dysbiosis during RT	features	ref
Ingrosso 2021	(i) Diversity of oral microbiome decreases at phase II and III. (ii) Fungal infection increases at phase III and IV (iii) A significant increase in Lactobacilli is found at phase III and IV.	(33)
Vesty 2020	(i) The salivary microbiota remain stable during RT and are consistently dominated by Streptococcus, Prevotella, Fusobacterium and Granulicatella. Obligate and facultative anaerobic Gram-negative bacilli (GNB) Bacteroidales G2, Capnocytophaga, Eikenella, Mycoplasma and Sneathia, as well as anaerobic GNB in the periopathogenic genera Porphyromonas and Tannerella, were all positively correlated with ≥ grade 2 OM. (ii) Significant increases in the relative abundances of Bacteroidales G2, Fusobacterium and Sneathia were identified in buccal mucosa swabs at sites of ≥ grade 2 OM	(36)
Zhu 2017	(i) Bacterial community structure altered progressively during RT, in parallel with a marked increase in the relative abundance of some Gram-negative bacteria. (ii) Patients who eventually developed severe mucositis harbored a significantly lower bacterial alpha diversity and higher abundance of Actinobacillus during the phase of erythema - patchy mucositis.	(34)
Hu 2013	(i) The microbial composition of oral plaques changes during HN-RT. (ii) A negative correlation between the number of operational taxonomic units (OTUs) and radiation dose was found.	(35)

II. MATERIALS AND METHODS

Study design for obtaining oral microbiome data during radiation therapy

Patients were scheduled to receive radiation therapy for head and neck squamous cell carcinoma at the Department of Dentistry and Oral Surgery, Fukuyama City Hospital. Supplementary figure S1 outlines the participant information.

Samples were collected to obtain microbiome data during radiation therapy using 16S ribosomal DNA sequencing. Three patients with oral squamous cell carcinoma participated in this microbiome study during radiation therapy for head and neck cancer treatment. Oral irrigation samples were collected at pre-RT, 20 Gy, 40 Gy, 50 Gy, and post-RT (one month after radiation treatment) to evaluate microbiological changes (Supplementary fig. S1).

At the time of sampling, medical information was obtained from oral examinations and medical records. The severity of mucositis was evaluated according to the World Health Organization (WHO) scale, 0-4, which assesses oral soreness, erythema, and ulceration (https://www.statpearls.com/ArticleLibrary/viewarticle/19378).

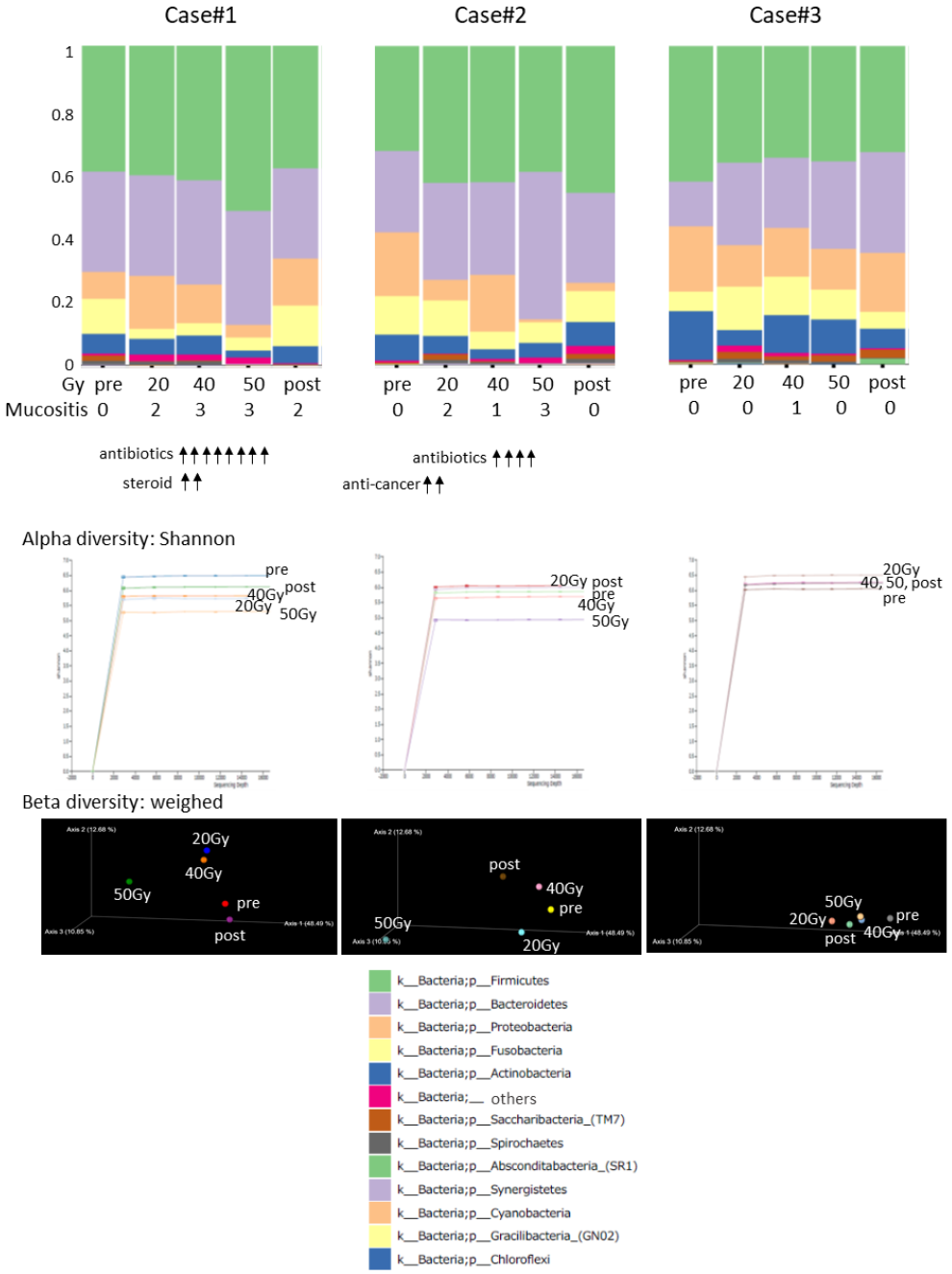


Fig. 1. Change in microbiome composition in the mouth during radiation therapy to treat head and neck cancer.

The relative abundance of the predominant bacterial taxa at the phylum level (level 2) is shown for the three participants. The bacterial enrichment was changed at a 50 Gy-irradiation dose in cases #1 and #2, who were administered antibiotics and other medications. In case #3, systemic drugs were not administered during radiotherapy, except for local ointments. The Shannon diversity index is shown as the alpha diversity. Beta diversity represents a comparison of the bacterial ratios in each microbiome.

A score of 0 indicated no signs or symptoms, 1 indicated erythema and soreness, 2 indicated ulcers, able to eat solids, 3 indicated ulcers, required liquid diet (due to mucositis), and 4 indicated ulcers, alimentation was not possible (due to mucositis). This study was approved by the ethics committee of the Fukuyama City Hospital (No.544). Informed consent was obtained from all participants using a written contract.

Sample collection from patients during radiation therapy

In general, the oral microbiome is variable and has different colonization sites in the mouth [37]. Because sampling procedures can affect the results, sampling is one of the most important steps that impact whether accurate information on the oral microbiome is obtained. In this study, oral conditions changed depending on the irradiation dose administered. We predicted that participants would suffer from xerostomia during radiation therapy due to the hypofunction of irradiated salivary glands. Therefore, we decided to collect a saline irrigation solution with 0.9% saline to evaluate the microbiome. Participants were instructed to avoid eating or drinking anything other than water 1 h before sample collection. Saline irrigation solution was collected after adding 20 ml of saline solution to the whole mouth using a disposable syringe. Participants shake their heads from side to side and rinse the mouth to disseminate saline solution into the whole mouth and spit the solution into a sterile tray. The collected samples were transferred into a sterile 50 ml tube and stored at −80°C.

Saliva sampling for amplification of oral flora from a healthy volunteer

Saliva was obtained from a healthy volunteer who provided informed consent [38]. Saliva sampling was approved by the Ogaki Women's College Ethical Research Committee (Epidemiology-No.R1-5/R2-1)

The volunteer had not taken any medication for 3 months before the study and did not use any chemical mouthwashes 3 h before sampling. Eating and drinking were not permitted for 2 h before sampling. Culturing/amplification of oral flora was performed by adding 10% supplements (Supplementary Table S1). Supplements used included brain heart infusion (BHI) broth (a bacterial nutrient-rich medium), Dulbecco's modified Eagle medium (a tissue culture medium), 0.22 μm-filtered saliva from the volunteer to remove microbiota (the organic components of saliva), and by addition of Saliveht, i.e., artificial saliva (inorganic components of saliva, Teijin). Culturing/amplification was performed at 37°C in aerobic conditions for 24 and 48 h. BHI is a nutrient-rich medium for bacterial growth, which enables the fast growth of oral bacteria. Among the four supplements, BHI was used to evaluate the microbiome of the sample at 8, 24, and 48 h.

In the second experiment, to analyze the dose-dependency of supplements, culturing/amplification was performed at 37°C for 48 h in aerobic conditions at concentrations of 0.5%, 5%, and 50% (Supplementary Table S2).

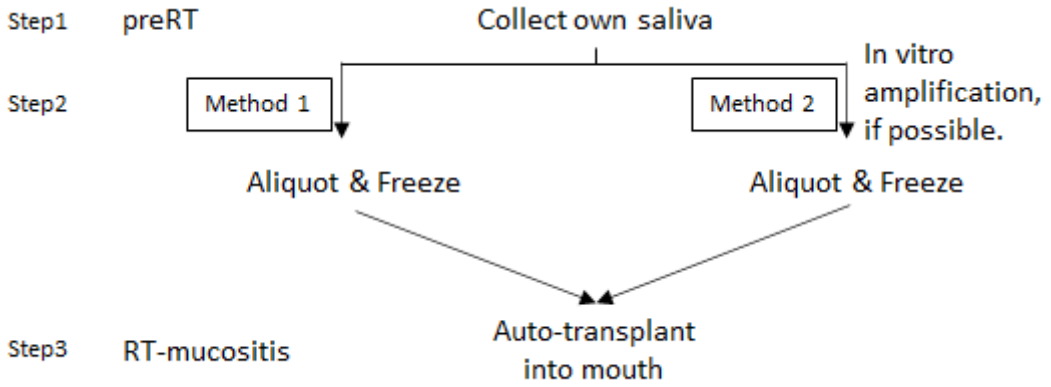


Fig. 2. A hypothesis on the control of the oral ecosystem.

Data for microbiome analysis during radiotherapy of head and neck cancer showed that a cumulative dose of 20–30 Gy-irradiation initiates oral mucositis; concomitantly, the bacterial ecosystem changes in the mouth. We hypothesized that the original oral flora present in the patient before radiation therapy is an original ecosystem that can be used in auto-transplantation to treat dysbiosis. Before radiation therapy, the patient can collect saliva and stock it in the freezer. After radiation-mucositis occurs, the aliquoted ice cube of the stored

saliva can be returned to the mouth. This may help correct dysbiosis, and ice cubes cool the mouth. In addition, the administration of saliva reduces hyposalivation.
In method 2, we propose the potential application of *ex vivo* amplification of the oral flora. We aimed to culture/amplify the oral flora without changing the bacterial ratio. If this can be enabled, the amount of saliva required for collection by the patient can be reduced.

DNA preparation

Bacterial DNA was extracted from oral irrigation samples using the QIAamp UCP Pathogen Mini Kit (Qiagen). Bacterial pellets were suspended in 500 µL of ATL buffer provided with the kit. Bacterial pellets were transferred to a Pathogen Lysis Tube S (Qiagen) and homogenized with a Mixer Mill MM 301 (Retsch) for 3 min at a vibrational frequency of 30 Hz. After elution with 50 µL of AVE buffer, DNA was quantified using a Quantus fluorometer (Promega) and a Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific) and stored at –80°C until use.

Library construction and high throughput DNA sequencing

PCR of prokaryotic 16S ribosomal DNA and library construction was performed according to the 16S Metagenomic Sequencing Library Preparation guide supplied by Illumina (part No. 15044223_B). Briefly, the V3–V4 region of the 16S ribosomal RNA gene was amplified via polymerase chain reaction (PCR) using 341f/806r primers and Premix Ex Taq polymerase (Takara Bio). The PCR amplicons were purified using AMPure XP beads (Beckman Coulter). Sequencing adapters containing 8 bp indices were incorporated into the 3′ and 5′ ends of the purified amplicons by PCR. The PCR products were repurified with AMPure XP beads and quantified using a Quantus fluorometer. After pooling equimolar amplicon concentrations, a 5% equimolar amount of PhiX DNA (Illumina) was added, and the resulting library was paired-end sequenced using a read-length of 2 × 250 bp with a MiSeq Reagent Kit v2 on the Illumina MiSeq platform.

Processing of sequences and taxonomic classification

The 16S rDNA gene reads were processed as described [39]. Primers and barcodes were trimmed, followed by removal of sequences shorter than 200 bp, along with homopolymers greater than eight nucleotides or ambiguous base calls. Sequences were filtered using a 50 bp sliding window approach and an average quality score threshold of 35 [39]. Chimeric sequences were removed using UChime [40]. Sequences were classified to species level using the classify.seqs command and the Human Oral Microbiome Database (HOMD) V14.5. The taxonomy assignment algorithm was validated by classifying HOMD reference sequences trimmed to include the V1–V2 region alone against the HOMD full-length reference sequence database.

ITS-1 reads were processed as described [39]. Sequences were trimmed and quality filtered, and chimeras were removed as described for 16S rRNA sequences. Sequences were then classified at the genus level using the classify.seqs command and a modified version of the Findley's database [41] as a reference, with the recommended parameters (https://www.mothur.org/wiki/Findley_ITS_Database). Before classification was performed, a curation of Findley's database was conducted to identify synonymous taxa under one preferred name [42]. To accomplish this, reference sequences from synonymous taxa were compared using BLAST against the NCBI nucleotide type strain database.

Data analysis

The sequences were analyzed using the CLC Workbench software (Version 10.0.1; Filgen) equipped with the Microbial Genomics Module plugin (Version 2.0; Filgen). Quality and chimera crossover filtering was performed using default parameters. Operational taxonomic unit (OTU) clustering and taxonomic assignment were performed using the Human Oral Microbiome Database (Version 14.51) as a reference (clustered at 97%). Low-abundance OTUs were discarded (less than 10 reads; less than 0.01% relative abundance). The Shannon index and UniFrac distance were used to evaluate alpha and beta diversity, respectively [43]. All analyses, from chimera checking to alpha and beta diversity calculations, were performed using QIIME (1.9.1) [44].

qPCR

To evaluate bacterial numbers in each sample after culturing/amplification of oral flora, quantitative PCR was performed using the quantitative *tuf* PCR kit for bacteria (Takara Bio, Shiga, Japan) with 1 µL of template DNA in a total volume of 25 µL. The reaction mixture was prepared according to the manufacturer's instructions. The amplification program included an initial denaturation step of 30 s at 95°C, followed by 35 cycles of 5 s at 95°C, and 30 s at 60°C. Simultaneously melting curve analysis was performed. Negative controls were included in each experiment. Amplification was performed on a StepOne and StepOnePlus Real-Time PCR System (Thermo Fischer Science, Tokyo, Japan). All amplification reactions were performed in

triplicates. A positive *tuf* gene standard stock solution provided in the kit was used to generate a linear standard curve.

Reagents used in this study

BHI broth, brain heart infusion (8.0 g), peptic digest of animal tissue (5.0 g), pancreatic digest of casein (16.0 g), dextrose (2.0 g), sodium chloride (5.0 g), and disodium phosphate (2.5 g) per liter were obtained from BD. Dulbecco's modified Eagle's medium (DMEM), 4500 mg/L glucose, 1 mM sodium pyruvate, 1500 mg/L sodium bicarbonate, and 4 mM L-glutamine were prepared using reagents obtained from Merck.

Saliveht (Saliveht Aerosol, artificial inorganic saliva); sodium chloride (42.2 mg), potassium chloride (60.0mg), calcium chloride hydrate (7.3 mg), magnesium chloride (2.6 mg), dipotassium phosphate (17.1 mg/50 g), pH 5.0–6.0 (Teijin).

Human serum and heat-inactivated human serum from male AB plasma were obtained from Sigma-Aldrich.

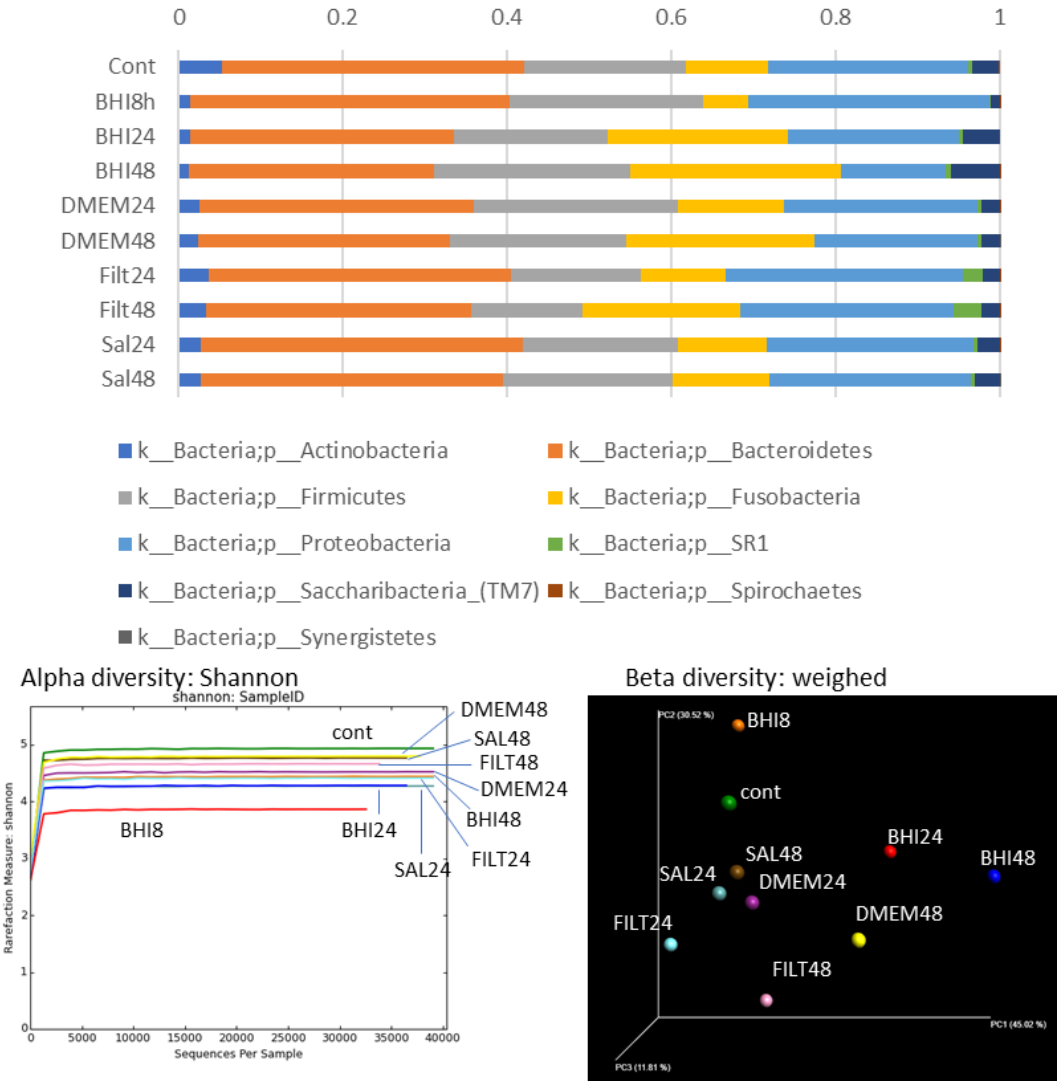


Fig. 3. *Ex vivo* culturing/amplification of oral flora.

A volunteer's saliva sample was used to culture/amplify the oral flora under several conditions. The conditions included adding (i) brain heart infusion medium (BHI), one a bacterial broth, (ii) Dulbecco's modified Eagle's medium (DMEM), a tissue culture medium, (iii) Saliveht (SAL), an artificial saliva, which is composed of inorganic salivary molecules, (iv) the patient's own organic saliva filtered through a 0.22 µm membrane to remove microorganisms (FILT). These supplements were added to the saliva at a concentration of 10% (for example, 220 µl supplement/2 ml saliva), and incubated in culture tubes in standing condition at 37°C for 24 and 48 h. Because BHI is a nutrient-rich medium, samples after 8 h of culture were checked for early amplification.

After culturing, the microbiome was compared with that in control saliva, which was immediately frozen after sampling. Beta diversity analysis showed that the microbiome composition from the 48 h culture with inorganic saliva (artificial saliva, SAL) showed the greatest similarity to the control.

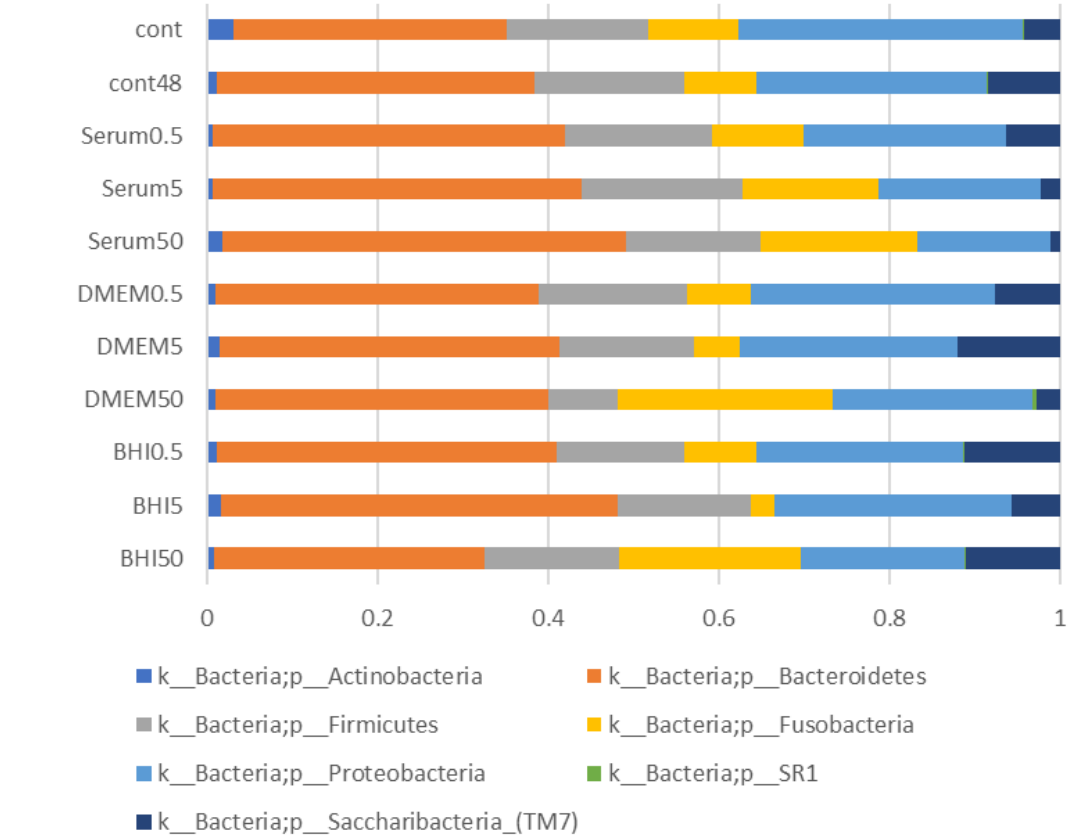
III. RESULTS

The occurrence of dysbiosis during RT

NGS techniques are used worldwide, and useful data on the oral microbiome have been accumulated and published by several organizations, such as eHOMED (http://www.homd.org/). Data on the microbiome during RT have also been reported (Table 1). Therefore, we focused on the clinical reality of RT for head and neck cancers. For example, during RT, physicians may have to administer systemic steroids during care for the patient. Additionally, physicians may need to administer antibiotics to prevent focal infection from a small periapical lesion during RT. We included patients administered various treatments to study the microbiome and its changes during RT. Although three participants were included in the study and the sample size is small, the clinical features of the microbiome during RT can still be examined.

As shown in fig. 1, RT-OM (oral mucositis) was diagnosed in all cases during RT. The relative abundance of predominant bacterial taxa at level 2 (phylum) indicated the prevalence of microbial diversity among the three participants. We evaluated the effects of radiation dose, and bacterial enrichment changed at an irradiation dose of 50 Gy in cases #1 and #2. Medical records showed that systemic administration of antibiotics was performed during this period to prevent focal infection from silent periapical lesions. The Shannon diversity index analysis indicated the prevalence of reduced diversity after administration of an irradiation dose of 20~50 Gy in case #1. Case#2 was administered an anti-cancer chemotherapy drug and antibiotics during 20~50 Gy irradiation. In these cases, the microbial profiles between preRT and postRT were similar, suggesting that the postRT microbiome returned to that observed during preRT. In case #3, systemic drugs were not administered during RT. The results showed that alpha diversity was similar during RT.

Beta diversity analysis is performed to characterize the bacterial ratios in each microbiome. In cases #1 and #2, using irradiation doses of 20 Gy and 50 Gy, postRT sample analysis showed low similarity for beta diversity to the control preRT sample. This may result due to systemic drug administration such as antibiotics, which may affect the microbiome composition. In case #3, at doses of 20 Gy and 40 Gy, postRT samples showed low similarity for beta diversity to the control preRT sample. These data indicate that dysbiosis occurred to some degree in all patients who were provided treatment, including the administration of antibiotics during RT.



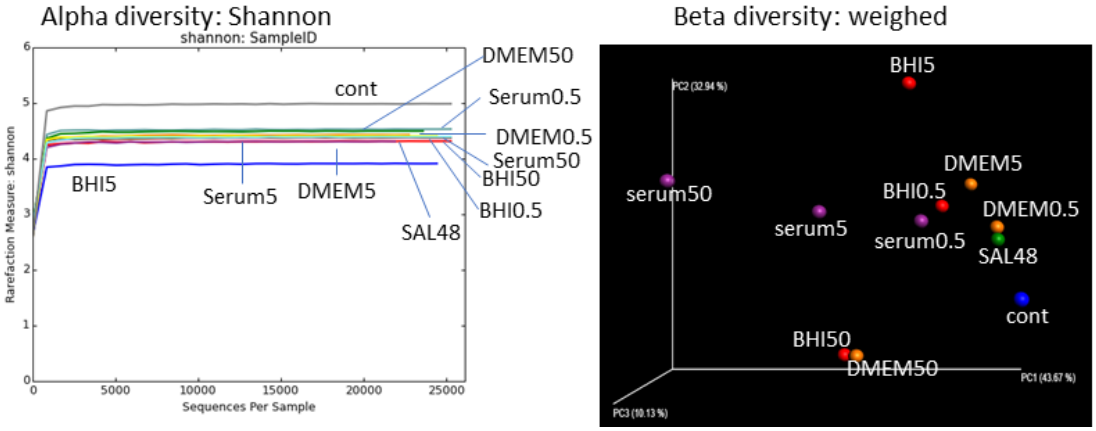


Fig. 4. Supplement-dependent conditions to culture/amplify oral flora.

To improve the culture conditions for amplifying oral flora, dose-dependent administration of supplements (0.5, 5, 50%) including brain heart infusion (BHI), DMEM, human serum (serum), and the artificial saliva Saliveht (as 2nd control: cont48) was performed at 37°C for 48 h under aerobic conditions. The volume was adjusted to 2 ml by adding Saliveht. For example, a sample can consist of 0.5% BHI; i.e., 10 µl BHI/1 ml saliva, and 990 µl Saliveht. Beta diversity analysis revealed that the microbiome from inorganic saliva (artificial saliva, cont48) showed the greatest similarity to the control, with the next best result was obtained using 0.5% DMEM.

Hypothesis for control of the oral ecosystem

After performing microbiome analysis during radiotherapy of head and neck cancer, a cumulative dose of 20–30 Gy irradiation was found to initiate oral mucositis along with bacterial ecosystem changes in the mouth. Because of antibiotic administration, the oral flora may have been altered further. We hypothesized that the bacterial ecosystem prevalent in patients before radiation therapy is the original oral flora. Before radiation

therapy, the oral ecosystem that is prevalent can serve as a probiotic against dysbiosis, and a blueprint is shown in Fig.2. We hypothesized that the auto-transplantation of saliva with the so-called original oral flora may help in microbiome recovery and alleviation of mucositis symptoms. Before radiation therapy, the patient can collect their saliva and stock it in a freezer. Once radiation mucositis occurs, the aliquoted ice cube of the stored saliva is placed in the mouth. This may help correct the dysbiosis, and ice cubes can cool the mouth. In addition, the administration of saliva reduces hyposalivation. For example, one ice cube containing 3 ml of saliva can be put back into the mouth once a day, after the onset of oral mucositis. Physicians can choose between the administration of 1 ml of saliva 3 times per day, or a 3 ml ice cube once per day depending on the severity of OM. Usually, oral mucositis lasts approximately one month after administration of an irradiation dose of 20–60 Gy. Therefore, patients can stock their saliva (total 3 ml × 30 days = 90 ml) before the start of therapy.

Potential of *ex vivo* culturing/amplification of oral flora

Next, we examined whether it is possible to culture/amplify the oral flora from patients without changing the bacterial ratio. If this is possible, a reduced dose of saliva collection will be required. If a culturing/amplification method can be established, the original oral flora can be used as a probiotic for medical treatment. We aimed to culture/amplify the oral flora of volunteers under several conditions, including (i) adding brain heart infusion medium, one of the bacterial culture broths, (ii) adding Dulbecco's modified Eagle's medium (DMEM), a tissue culture media, (iii) adding artificial saliva, Saliveht, which is composed of inorganic salivary molecules; and (iv) adding the volunteer's saliva filtered through 0.22 μm membranes to remove microorganisms. These supplements were added to the saliva at a concentration of 10% and incubated in culture tubes in standing conditions at 37°C for 24 and 48 h. Because BHI is a nutrient-rich medium, data for 8 h were obtained from one sample alone using 10% BHI. After culturing/amplification, the microbiome was compared with the control saliva, which was immediately frozen at sampling. As shown in fig. 3, beta diversity analysis showed that the microbiome from SAL-48 h, i.e., inorganic saliva (artificial saliva, Saliveht) was the most similar to the control.

To improve conditions for the amplification of oral flora, dose-dependent challenges using supplements (0.5, 5, and 50%) were performed. In general, saliva is produced by acinic cells using serum from the blood. Human serum was added as a candidate supplement to culture/amplify oral flora. As shown in fig. 4, beta diversity analysis results showed that the microbiome from SAL48, which was cultured with the collected oral flora for 48 h with 50% Saliveht (inorganic artificial saliva), showed the most similar beta diversity to the control. This result was unexpected because 50%-Sal was used as a 2nd control for volume adjustment. The next best result was obtained using 0.5% DMEM.

qPCR was performed to determine the number of bacteria that were cultured/amplified under these conditions. As shown in fig. 5, qPCR results indicated that the bacterial numbers in the control sample were 6.95×10^7/ml. Bacterial numbers in the inorganic saliva (Saliveht) sample and the 0.5% DMEM sample were 2.28×10^8 and 2.24×10^8/ml, respectively. This result indicates that bacterial numbers were amplified 3.28 times and 3.22 times, respectively.

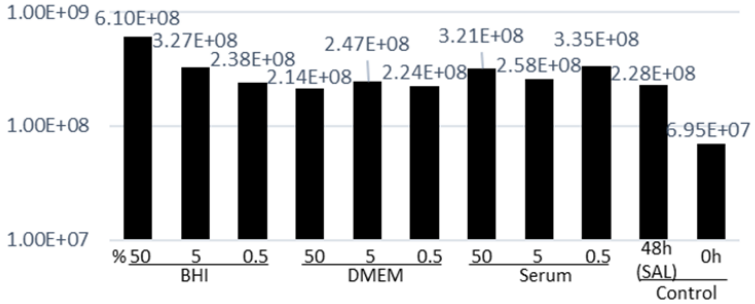


Fig. 5. qPCR to evaluate total bacterial numbers after *ex vivo* culturing/amplification of oral flora.

After culturing and amplification under several conditions, qPCR was performed. The results indicated that the bacterial numbers in the control sample were 6.95×10^7/ml. Bacterial numbers in inorganic saliva and 0.5% DMEM samples were 2.28×10^8 and 2.24×10^8/ml, respectively. Therefore, bacterial numbers were amplified at a ratio of 3.28 and 3.22 times, respectively.

IV. DISCUSSION

In this study, we studied the change in microbiome composition during RT, including situations where antibiotics and/or steroids were administered, as dysbiosis is implicated during RT-OM. Most Japanese physicians prefer prophylactic antibiotics to prevent or reduce focal infections and acute periodontitis in the opportunistic conditions provided by irradiated mouths. In general, physicians may be required to administer antibiotics, even when the signs of infection are not very strong, such as the presence of small gingival abscesses or slight pericoronitis of the wisdom tooth. Dysbiosis may occur due to host factors, such as hyposalivation, tissue damage, and immune cell dysfunction. Further, the sensitivity of oral flora to radiation is also a key factor in inducing microbiome changes. For example, *Deinococcus radiodurans* is a radioresistant bacterium, which can compensate for extensive DNA damage via the activity of DNA-repair proteins to avoid the potentially detrimental effects of DNA strand breaks [45-47]. *Deinococcus* is a genus of soil bacteria that are not found in oral flora; however, bacteria with a similar mechanism for radiotolerance may exist in the oral cavity. Alternately, radiation therapy may induce the proliferation of radioresistant bacteria. Further studies will be required to understand the resistance mechanisms involved.

We performed analysis on dysbiosis during HN-RT as summarized in table 1, and hypothesized that the ecosystem prevalent before radiation therapy is the original oral flora in patients. The oral ecosystem before radiation therapy may serve as a probiotic during the period of dysbiosis. Researchers that have proposed an association between oral dysbiosis and cancers may argue for administering patients' saliva into the mouth [8, 48]. It is unclear whether the oral microbiome affects the initiation and progression of oral cancer; however, data for oral cancer indicates that the prognosis for surgical treatment was not substantially different from other therapies and their combinations [49, 50]. The original oral flora from HN cancer may not have carcinogenic activity.

To carry out our blueprint shown in fig 2, three steps require implementation. In the first step, saliva should be collected from a patient before radiation therapy. Second, the collected saliva should be stored in a freezer. For freezing, a stock procedure is required, such as adding 10% glycerol. The third step and most important step requires the cooperation of participants and hospitals to perform clinical administration. Therefore, in this study, we focused on the second step of the blueprint. Culturing/amplification of oral flora may help maintain the bacterial content ratio. In this study, we investigated several culture conditions for maintaining the original bacterial composition. Unfortunately, nutrient-rich media amplifies several specific species. This may occur because viable but difficult to culture bacteria exist and may play a role in the oral ecosystem. Our results showed that inorganic artificial saliva or standing culture without any supplements can modestly enable the culture/amplification of oral flora. Although culturing/amplification methods are still in development, a report exists on culturing the fecal microbiome *ex vivo* [51]. The aim of the study was to culture gut flora was to establish a model mimicking the real stomach environment to study drug metabolism catalyzed by fecal flora. The authors used 14 different media to grow several species anaerobically over two days. They concluded that the modified GAM (Gifu anaerobic media) supported the growth of a bacterial community that was similar in composition and diversity to the control. The modified GAM contained peptone, soya peptone, proteose peptone, digested serum, yeast extract, meat extract, liver extract, dextrose, soluble starch, L-tryptophan, L-cysteine hydrochloride, sodium thioglycolate, l-arginine, vitamin K1, hemin, potassium dihydrogen phosphate, and sodium chloride. The composition is similar to ordinary nutrient-rich medium, and can be analyzed to answer why the modified GAM showed a good result for supporting the growth of several microbial species. Moreover, as the authors aimed to optimize an *ex vivo* mixed culturing system, the total bacterial numbers were not evaluated/shown. In our study, oral flora in saliva consisted mainly of facultative anaerobes; however, the culture conditions chosen were aerobic. The oral cavity is a food delivery route, and saliva flows and dilutes the food content; therefore, the oral flora lives in nutrient-poor circumstances. We did not use a modified GAM for our study. Enomoto et al. [52] studied saliva storage methods for microbiome analysis. They showed that incubation at 20°C for a few hours did not affect the overall microbiome structure. None of the media was added. This report supports the conclusion that Saliveht (artificial inorganic saliva) does culture/amplify oral flora without changing the bacterial ratio.

It can be debated whether ~3× amplification is meaningful or not. If amplification of oral flora is achieved, the dose of saliva required for collection can be reduced. As mentioned above, the collection of 90 ml of saliva, which should be obtained before radiation therapy, can be reduced to 30 ml by using standing saliva cultured with Saliveht at 37°C for 48 h. Saliveht is artificial saliva and consists of inorganic components, which is safer for transplantation into the body than media containing high molecular weight bioactive factors. Further studies are required to determine whether cultured/amplified bacteria can function as probiotics. Japanese law is strict concerning the analysis of new medical therapies; therefore, we propose collaboration with researchers who are interested in the hypotheses proposed in this study and can perform salivary transplants during radiation therapy for head and neck cancer.

Table 2. Hypothesis on benefit of salivary transplantation (transplantation of oral flora)

	donor	modification	recipient	Against lesions
Auto-transplantation	Saliva (oral flora) before radiation therapy on oral cancer	Frozen aliquot	Themselves after onset of radiation stomatitis	(i)Radiation stomatitis, (ii)Candidiasis (iii)Xerostomia-related dental caries
Allo-transplantation	Saliva (oral flora) from health human without any dental caries	Frozen aliquot	Kids during "Window of infectivity (mutans streptococci)"	Dental caries
	Saliva (oral flora) from health human without any dental caries	Amplification & powderization if possible	Kids during "Window of infectivity (mutans streptococci)"	Dental caries
	Caregivers' saliva (oral flora) regardless carrying dental caries	Removal of mutans streptococci by flowcytometer & cell sorting	Their own kids during "Window of infectivity (mutans streptococci)"	Dental caries
Artificial	Invitro preparation of ideal oral flora	Artificially –made super probiotics	Patients with oral problems	Dental caries, Perio?, candida

Several researchers have attempted to overcome the therapeutic challenges posed by RT-OM [33, 53]. Locally applied chemical agents include anesthetic ointments and artificial saliva. Systemically administered medications include non-steroidal anti-inflammatory drugs and antibiotics. The administration of probiotics using a few bacteria has been performed, especially using *Lactobacillus* species [54-57]. For example, the use of *Lactobacillus brevis* CD2 to reduce oral mucositis in randomized studies involving patients with head and neck cancers has been studied [54]. *Lactobacillus brevis* CD2 decreases inflammation by reducing arginine bioavailability. In a mouse model, *Lactobacillus reuteri* DSM17938 improved oral inflammation by increasing epithelial thickness and reducing oxidative stress [56]. Live cells and cell-free supernatants from *Lactobacillus paracasei* 28.4 cultures can inhibit the growth of *Candida auris*, suggesting the prevalence of probiotic and postbiotic effects [58]. To analyze exacerbation of the dental cavity and periodontitis during radiotherapy, *Streptococcus salivarius* M18 was used in patients with head and neck cancer who underwent radiotherapy [59]. An improvement in periodontal screening results and plaque index scores were observed, which may occur because of host immune response modulation and microbial interactions through antagonism and co-aggregation. In general, oral probiotics should colonize at appropriate sites in coordination with commensal oral flora. It is also unclear how additional bacteria and their components behave in the mouth and the gastrointestinal tract, and subsequently in the body. In our blueprint, this problem may not be of significance, because the proposed probiotics originate from the patients' own oral flora. However, freezing and culturing/amplification may produce unfamiliar metabolites. Culturing with artificial saliva (inorganic saliva) showed the best results, and the side effects associated with artificial inorganic saliva are rare. Moreover, the oral flora of patients can easily colonize at appropriate sites in their own mouths, forming their own ecosystems. Collaboration to perform auto-transplantation during RT is the next step in this study.

Although we propose that the use of patients' own oral flora may be ideal, the definition of "ideal oral flora" is ambiguous. The establishment of an "individual ecosystem" or "functional ecosystem" is the short-term goal of our study. Additionally, we propose auto-transplantation of oral flora and allotransplantation of the mother's oral ecosystem with some modifications. We hypothesize that these procedures can prevent caries (Table 2). Two important steps are required to be implemented: collecting oral flora from caries-free volunteers who are not fluoride-treated and are not on a sugar-based diet. Second, the collected oral flora can be used as probiotics and transplanted into the mouth of children during the window of infectivity. A modified method of caries prevention is also proposed using probiotics from the mother, regardless of the presence of dental caries in her mouth [60]. In previous studies, we have reported that a flow cytometry-based cell sorter can separate *Streptococcus mutans* from oral flora using fluorescence-conjugated anti-*S. mutans* antibodies. After bacterial sorting by flow cytometry, the mother's oral flora without *S. mutans* can be transplanted into the mouth of children during the window of infectivity. This research is awaiting a clinical trial.

The amplification of oral flora without changing the microbiome is a difficult process. If successful, this method may be applicable for treating the gut/intestine microbiome. We hope to help patients with dysbiosis by improving their oral ecosystems.

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We declare no conflict of interest in this study.

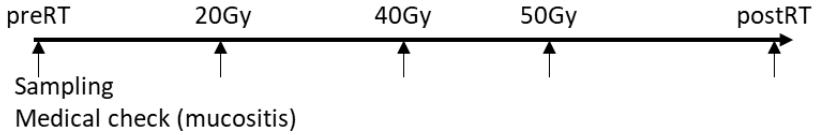
REFERENCES

[1]. Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner ACR, Yu W, Lakshmanan A, Wade WG. The human oral microbiome. J Bacteriol. 2010; 192: 5002–17.
[2]. Munyaka PM, Khafipour E, Ghia J-E. External influence of early childhood establishment of gut microbiota and subsequent health implications. Front Pediatr. 2014; 2(109): 1-9.
[3]. Gomez A, Nelson KE. The Oral microbiome of children: Development, disease, and implications beyond oral health. Microb Ecol. 2017; 73(2): 492-503.
[4]. Costello EK, Stagaman K, Dethlefsen L, Bohannan BJ, Relman DA. The application of ecological theory toward an understanding of the human microbiome. Science. 2012; 336:1255–62.
[5]. Lif Holgerson P, Öhman C, Rönnlund A, Johansson I. Maturation of oral microbiota in children with or without dental caries. PLoS One. 2015; 10: 1-20.
[6]. Gupta VK, Paul S, Dutta C. Geography, Ethnicity or subsistence-specific variations in human microbiome composition and diversity. Front Microbiol. 2017; 8(1162): 1-16.
[7]. Caselli E, Fabbri C, D'Accolti M, Soffritti I, Bassi C, Mazzacane S, et al. Defining the oral microbiome by wholegenome sequencing and resistome analysis: the complexity of the healthy picture. BMC Microbiol. 2020; 20(120): 1-19.
[8]. Irfan M, Delgado RZR, Frias-Lopez J. The oral microbiome and cancer. J Front Immunol. 2020; 11(591088): 1-18.
[9]. Verma D, Garg PK, Dubey AK. Insights into the human oral microbiome. Arch Microbiol. 2018; 200(4): 525-40.
[10]. Yamashita Y, Takeshita T. The oral microbiome and human health. J Oral Sci. 2017; 59 (2): 201-6.
[11]. Dashper SG, Mitchell HL, Lê Cao KA, Carpenter L, Gussy MG, Calache H, et al. Temporal development of the oral microbiome and prediction of early childhood caries. Sci Rep. 2019; 9(1): 1-12.
[12]. Hurley E, Barrett MPJ, Kinirons M, Whelton H, Ryan CA, Stanton C, et al. Comparison of the salivary and dentinal microbiome of children with severe-early childhood caries to the salivary microbiome of caries-free children. BMC Oral Health. 2019; 19(13): 1-14.
[13]. Twetman S. Prevention of dental caries as a non-communicable disease. Eur J Oral Sci 2018; 126(Suppl): 19-25.
[14]. Kilian M. The oral microbiome - friend or foe? Eur J Oral Sci. 2018; 126(Suppl 1): 5-12.
[15]. Hajishengallis E, Parsaei Y, Klein MI, Koo H. Advances in the microbial etiology and pathogenesis of early childhood caries. Mol Oral Microbiol. 2017; 32(1): 24-34.
[16]. Lamont RJ, Koo H, Hajishengallis G. The oral microbiota: dynamic communities and host interactions. Nat Rev Microbiol. 2018; 16(12): 745-59.
[17]. Stone VN, Xu P. Targeted antimicrobial therapy in the microbiome era. Mol Oral Microbiol. 2017; 32(6): 446-54.
[18]. Costalonga M, Herzberg MC. The oral microbiome and the immunobiology of periodontal disease and caries. Immunol Lett. 2014; 162(2 Pt A): 22-38.
[19]. Wade WG. The oral microbiome in health and disease. Pharmacol Res. 2013; 69(1): 137-43.
[20]. Graves DT, Corrêa JD, Silva TA. The oral microbiota is modified by systemic diseases. J Dent Res. 2019; 98(2): 148-56.
[21]. Kleinstein SE, Nelson KE, Freire M. Inflammatory networks linking oral microbiome with systemic health and disease. J Dent Res. 2020; 99(10): 1131-9.
[22]. Sampaio-Maia B, Caldas IM, Pereira ML, Pérez-Mongiovi D, Araujo R. The oral microbiome in health and its implication in oral and systemic diseases. Adv Appl Microbiol. 2016; 97: 171-210.
[23]. Jensen SB, Vissink A, Limesand KH, Reyland ME. Salivary gland hypofunction and xerostomia in head and neck radiation patients. J Natl Cancer Inst Monogr. 2019; 2019(53): 95-106.
[24]. Moreno AC, Frank SJ, Garden AS, Rosenthal DI, Fuller CD, Gunn GB, et al. Intensity modulated proton therapy (IMPT) - The future of IMRT for head and neck cancer. Oral Oncol. 2019; 88: 66–74.
[25]. Sroussi HY, Epstein JB, Bensadoun RJ, Saunders DP, Lalla RV, Migliorati CA, et al. Common oral complications of head and neck cancer radiation therapy: mucositis, infections, saliva change, fibrosis, sensory dysfunctions, dental caries, periodontal disease, and osteoradionecrosis. Cancer Med. 2017; 6(12): 2918–31.
[26]. Reyes-Gibby CC, Wang J, Zhang L, Peterson CB, Do KA, Jenq RR, et al. Oral microbiome and onset of oral mucositis in patients with squamous cell carcinoma of the head and neck. Cancer. 2020; 126(23): 5124-36.
[27]. Hollingsworth BA, Cassatt DR, DiCarlo AL, Rios CI, Satyamitra MM, Winters TA, et al. Acute radiation syndrome and the microbiome: Impact and review. Front Pharmacol. 2021; 12: 1-24.
[28]. Sami A, Elimairi I, Stanton C, Ross RP, Ryan CA. The role of the microbiome in oral squamous cell carcinoma with insight into the microbiome-treatment axis. Int J Mol Sci. 2020; 21(8061): 1-29.
[29]. Anjali K, Arun AB, Bastian TS, Parthiban R, Selvamani M, Adarsh H. Oral microbial profile in oral cancer patients before and after radiation therapy in a cancer care center - A prospective study. J Oral Maxillofac Pathol. 2020; 24(1): 117-24.
[30]. Mougeot JC, Stevens CB, Almon KG, Paster BJ, Lalla RV, Brennan MT, er al. Caries-associated oral microbiome in head and neck cancer radiation patients: a longitudinal study. J Oral Microbiol. 2019; 11(1586421): 1-13.
[31]. Arrifin A, Heidari E, Burke M, Fenlon MR, Banerjee A. The effect of radiotherapy for treatment of head and neck cancer on oral flora and saliva. Oral Health Prev Dent. 2018; 16(5): 425-9.
[32]. Yamashita K, Ohara M, Kojima T, Nishimura R, Ogawa T, Hino T, et al. Prevalence of drug-resistant opportunistic microorganisms in oral cavity after treatment for oral cancer. J Oral Sci. 2013; 55(2): 145-55.
[33]. Ingrosso G, Saldi S, Marani S, Wong AYW, Bertelli M, Aristei C, et al. Breakdown of symbiosis in radiation-induced oral mucositis. J Fungi (Basel). 2021; 7(290): 1-14.
[34]. Zhu XX, Yang XJ, Chao YL, Zheng HM, Sheng HF, Liu HY, et al. The potential effect of oral microbiota in the prediction of mucositis during radiotherapy for nasopharyngeal carcinoma. EBioMedicine. 2017; 18: 23-31.
[35]. Hu Y-J, Shao Z-Y, Wang Q, Jiang Y-T, Ma R, Tang Z-S, et al. Exploring the dynamic core microbiome of plaque microbiota during head-and-neck radiotherapy using pyrosequencing. PLoS ONE. 2013; 8(2): 1-7.

[36]. Vesty A, Gear K, Biswas K, Mackenzie BW, Taylor MW, Douglas RG. Oral microbial influences on oral mucositis during radiotherapy treatment of head and neck cancer. Support Care Cancer. 2020a; 28(6): 2683-91.
[37]. Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE. Defining the normal bacterial flora of the oral cavity. J Clin Microbiol. 2005; 43: 5721–32.
[38]. Hara K, Ohara M, Hayashi I, Hino T, Nishimura R, Iwasaki Y, et al. The green tea polyphenol (-)-epigallocatechin gallate precipitates salivary proteins including alpha-amylase: biochemical implications for oral health. Eur J Oral Sci. 2012; 120(2): 132-9.
[39]. Schloss PD, Gevers D, Westcott SL. Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. PloS One. 2011; 6(12): 1-14.
[40]. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics. 2011; 27(16): 2194–200.
[41]. Findley K, Oh J, Yang J, Conlan S, Deming C, Meyer JA, et al. Topographic diversity of fungal and bacterial communities in human skin. Nature. 2013; 498(7454): 367–70.
[42]. Diaz PI, Dupuy AK, Abusleme L, Reese B, Obergfell C, Choquette L, et al. Using high throughput sequencing to explore the biodiversity in oral bacterial communities. Mol Oral Microbiol. 2012; 27(3): 182–201.
[43]. Lozupone C, Knight R. UniFrac: a new phylogenetic method for comparing microbial communities. Appl Environ Microbiol. 2005; 71(12): 8228-35.
[44]. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010; 7(5): 335-6.
[45]. Bruckbauer ST, Cox MM. Experimental evolution of extremophile resistance to ionizing radiation. Trends Genet. 2021; 37(9): 830-45.
[46]. Mattimore V, Battista JR. Radioresistance of *Deinococcus radiodurans*: functions necessary to survive ionizing radiation are also necessary to survive prolonged desiccation. J Bacterial. 1996; 178(3): 633–637.
[47]. Cox MM, Battista JR. *Deinococcus radiodurans*— the consummate survivor. Nat Rev Microbiol. 2003; 3: 882–92.
[48]. Deo PN, Deshmukh R. Oral microbiome and oral cancer - The probable nexus. J Oral Maxillofac Pathol. 2020; 24(2): 361-7.
[49]. Kim B-G, Kim J-H, Kim M-I, Han JJ, Jung S, Kook M-S, et al. Retrospective study on factors affecting the prognosis in oral cancer patients who underwent surgical treatment only. Maxillofac Plast Reconstr Surg. 2016; 38(3): 1-9.
[50]. Wolfensberger M, Zbaeren P, Dulguerov P, Mu¨ller W, Arnoux A, Schmid S. Surgical treatment of early oral carcinoma—results of a prospective controlled multicenter study. Head Neck. 2001; 23(7): 525-30.
[51]. Javdan B, Lopez JG, Chankhamjon P, Lee YJ, Hull R, Wu Q, Wang X, Chatterjee S, Donia MS. Personalized mapping of drug metabolism by the human gut microbiome. Cell. 2020; 181(7): 1661–79.
[52]. Enomoto A, Nambu T, Mashimo C, Taniguchi M, Nakatani K, Komuro A, et al. A preliminary study of the effect of room temperature incubation on phylogenetic composition of salivary microbiota. J Oral Sci Rehab. 2018; 4(3): 1-7.
[53]. Villa A, Sonis ST. Mucositis: pathobiology and management. Curr Opin Oncol. 2015; 27(3): 159-64.
[54]. Sharma A, Rath GK, Chaudhary SP, Thakar A, Mohanti BK, Bahadur S. *Lactobacillus brevis* CD2 lozenges reduce radiation- and chemotherapy-induced mucositis in patients with head and neck cancer: A randomized double-blind placebo-controlled study. Eur J Cancer. 2012; 48: 875–81.
[55]. Zelante T, Iannitti RG, Cunha C, De Luca A, Giovannini G, Pieraccini G, et al. Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity via interleukin-22. Immunity. 2013; 39: 372–85.
[56]. De Sanctis V, Belgioia L, Cante D, La Porta MR, Caspiani O, Guarnaccia R, et al. *Lactobacillus brevis* CD2 for prevention of oral mucositis in patients with head and neck tumors: A multicentric randomized study. Anticancer Res. 2019; 39: 1935–42.
[57]. Gupta N, Ferreira J, Hong CHL, Tan KS. *Lactobacillus reuteri* DSM 17938 and ATCC PTA 5289 ameliorates chemotherapy-induced oral mucositis. Sci Rep. 2020; 10(16189): 1-11.
[58]. Rossoni RD, de Barros PP, Mendonca IDC, Medina RP, Silva DHS, Fuchs BB, et al. The postbiotic ativity of *Lactobacillus paracasei* 28.4 against *Candida auris*. Front Cell Infect Microbiol. 2020; 10(397): 1-15.
[59]. Vesty A, Gear K, Boutell S, Taylor MW, Douglas RG, Biswas K. Randomised, double‐blind,placebo‐controlled trial of oral probiotic *Streptococcus salivarius* M18 on head and neck cancer patients post‐radiotherapy: a pilot study. Sci Rep. 2020b; 10(13201): 1-9.
[60]. Ohara M, Hayashi I, Oda Y, Furutani C, Obayashi T, Nishi H, et al. Removal of mutans streptococci from saliva to establish non-cariogenic oral flora -A first step on exploring a new method using flow cytometry and cell sorting-. IJRES. 2017; 5(12): 41-54.

	gender	age	diagnosis
Case#1	female	41	Rt-tongue Ca (SCC)
Case#2	female	45	Lt-lower gingival Ca (SCC)
Case#3	male	63	Rt-upper gingival Ca (SCC)

Abbreviations Rt: right, Lt: left, Ca: carcinoma, SCC: squamous cell carcinoma



Supplementary Fig. S1. Schematic representation of oral microbiome analysis after radiation therapy for treating head and neck cancer.

The upper table shows the information for participants in the study. Microbiome analysis was scheduled as indicated by the lower arrows.

	8h	24h	48h
BHI	O	O	O
DMEM	-	O	O
Filt.saliva (FILT)	-	O	O
Saliveht (SAL)	-	O	O

O: performed, -: not performed
Supplements were added at concentration of 10%.
For example, 0.22ml supplement/2ml saliva

Supplementary Table S1. Culture/amplification conditions after the addition of 10% supplements.

To culture/amplify oral flora without changing the bacterial ratio, 10% of brain heart infusion (BHI), DMEM, 0.22 μm-filtered human saliva (saliva with organic components, FILT), and Saliveht, artificial saliva (inorganic components of saliva, SAL) were used as supplements for culturing/amplification at 37°C for 24 and 48 h under aerobic conditions. Supplements were added to a final concentration of 10%; for example, 220 μl supplement/2 ml saliva could be used. Because BHI is a nutrient-rich medium, samples after 8 h of culture were checked for early amplification.

	0.5%	5%	50%
BHI	O	O	O
DMEM	O	O	O
Serum	O	O	O
Saliveht	-	-	2nd control (cont48)

O: performed, -: not performed
Supplements were added at various concentrtions. A volume was adjusted to 2ml by adding saliveht.
For example, 0.5%BHI : 10μl BHI/1ml saliva/ 990μl saliveht

Supplementary Table S2. Concentration of supplements used for 48 h culture/amplification.

To culture/amplify oral flora without changing the bacterial ratios, various concentrations of brain heart infusion (BHI), DMEM, human serum (serum), and artificial saliva, Saliveht (as 2nd control: cont48) were used as supplements for culturing/amplification at 37°C for 48 h under aerobic conditions. The total volume was adjusted to 2 ml by adding Saliveht. For example, to make 0.5% BHI, 10 μl BHI/1 ml saliva was prepared in 990μl Saliveht.