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Removal of mutans Streptococci From SalivaTo Establish Non-Cariogenic Oral FloraA First Step on Exploring A New Method Using Flow Cytometry And Cell Sorting

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ABSTRACT: Dental caries is still a major problem in public health. Among many strategies, water fluoridation is recognized as an effective prophylaxis. However, this method has recently declined probably due to the adverse effect. We proposed a new procedure to prevent dental caries in children, using flow cytometry (FCM) and cell sorting system. At first, mutans streptococci can be removed from caregiver's (mainly mother's) oral flora. Second,oral flora without cariogenic bacteria will be transplanted into children at the period of "window of infectivity".Here we studied initial step on this blueprint.FCM analysis clearly separated laboratory Streptococcusmutans using rabbit anti-Strep. mutans IgG (Ab). Cross-reactivity of this antibody occurred to Strep. sobrinusas positive adverse effect, and Strep. salivarius as negative effect. FCM also revealed that mutans streptococci was separated from other oral flora of total four volunteers' saliva though it may be contaminated with Strep. salivarius to some degree. FCM and cell sorting system divided Strep. mutans Ab-positive F1 and -negative F2 fraction. Culture, quantitative PCR, and morphological observations confirmed relatively-good separation. Despite some problems, such as cross-reactivity of Ab, FCM and cell sorting system will be a good tool to separate mutans streptococci from oral flora.

Keywords: mutans streptococci, flow cytometry, cell sorting, oral flora

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I. INTRODUCTION

After raising the consumption of sugar in the food, human being are forced to fight painful dental caries in these decades (1).The Global Burden of Disease 2010 study estimated that 2.4 billion people were suffering from untreated cariesin the world (2). They also indicated that621 million children were affected with untreated caries in deciduous teeth.Dental caries is a complex multifactorial lesionincluding "Keyes triad": dietary sugar (diet), cariogenic bacteria (dental plaque), and susceptible teeth (host) (3, 4). In addition, NEWBRUN proposed"duration time"as the fourth factor in "Keyes triad"(1). Focused on these four factors, human being have been attempting many strategies to reduce the incidence of dental caries. Against dietary factor, many parents attempt no sucrose-diet to prevent dental caries from their children. They also want their children to do toothbrush and dental floss after every meal to remove food deposits from the teeth. Another simple method is a xylitol-diet as a substitute of sucrose. It is presumed that cariogenic bacteria cannot metabolize and utilize xylitol as the other sugars (5).Against susceptible host teeth, dental sealant is a good method to prevent dental caries, which coats the toth surface by thin dental resin. The most popular and effective method is systemic or topical application of fluorides (6). Fluoride dentifrices, rinses, and varnishes are the representatives of topical fluoride application.As of fluoridation of the water supply,approximately 370 million people drink artificially fluoridated water in approximately 27 countriesin the world until 2011 (7).Centers for Disease Control (CDC) in US proposed that water fluoridation was one of the top ten public health achievements of the 20th century (8). However, the caries preventionby water fluoridation have declined in the last decades probably due to that topical fluoride application became more popular and relatively effective(9). Moreover, enamel fluorosis has been reported as an emerging problem in fluoridated areas (10). In severe cases this can result in erosion of not only the teeth but also the skeletal bone (11). Therefore, controversial opinions from political, ethical, pharmacological, and safety concerns are still there on the water fluoridation (8, 12).

Against cariogenic bacteria, there are also many strategies attempted to combat these pathogens, such as vaccination (13, 14), specific anti-microbial agents (15), or inhibition of water insoluble glycan formation (16)and replacement therapy (17, 18). The most common bacteria associated with dental caries are the mutans streptococci, prominently Streptococcus mutans and Strep. sobrinus, orLactobacilli. Cariogenic bacteria use mainly sucrose or other dietary sugars provided from dental plaque to produce lactic acid or acetic acid through glycolytic cascade (19). These acids breakdown the teeth surface in the micro-environment covered by biofilm composed of water insoluble glycan chains polymerized by glucosyltransferases secreted by mutans streptococci (20).Mutans streptococci is a one of the oral commensal microorganisms (21). The oral cavity contains many different microorganisms comprised of over 600-700 species, which is called "Oral flora" (22). Oral flora is transferred into children from caregivers, predominantly their mothers (23). $C_{AUFIELD}$ monitored oral bacteria of 46 mother-child pairs from infant birth up to five years of age (24). The initial acquisition of mutans streptococci occurred in 38 children at the median age of 26 months during a discrete period were designated as the "window of infectivity". Although there was a report on late establishment of mutans streptococci in children, oral ecology including cariogenic bacteria can be established in these periods (25)We assumed that "window of infectivity" is only the period to artificially establish so-called "healthy oral flora", which means oral commensal microorganisms without cariogenic bacteria in their mouth. We propose the following two methods to establish the oral ecosystem as non-cariogenic environment. One is transplantation of saliva (oral flora) from healthy volunteers without any dental caries lesions to children's mouth at the period of "window of infectivity".Healthy volunteers without any dental caries are definedas human being not drinking fluoridated water or no-sugar dieting, but carrying oral ecosystem without cariogenic factors (26). If their oral ecosystem is established in children's mouth at "window of infectivity", transplanted children could never develop dental caries, just like the donor's mouth. In addition, there are options in this method. Allogenic salivary transplant is somehow cumbersome, because huge amount of no-caries volunteers' saliva can be required. Amplification, which means multiplication of non-cariogenic commensal microorganisms in vitro, should be performed, then, bred siblings will be transplanted into children.

It will be a medication or a prescription drugas caries preventionfor the children at "window of infectivity".The other is the modified method of the first one. Many parents probably hesitate to be transplanted complete stranger's saliva or its amplified oral flora into their beloved children. Therefore, if we can remove mutans streptococci from caregivers' (mainly mother's) saliva, parents' concern on unrelatedallo-transplant will be reduced, because vertical transplantation from caregiver's oral flora without cariogenic bacteria to their children can replace allo-transplantation. Fluorescence-activated cell sorting (FACS) using flow cytometry (FCM) was used to remove mutans streptococci from saliva. Here we propose the blueprint of vertical-transplant of saliva from caregiver to child. Because most of the commensal bacteria including in the mouth or in the colon are genetically identical between caregivers and children (18, 27). Our hypothesis and prophylaxis plan is shown on the blueprint in Fig.1. In this study, we attempted the first and second steps of this blueprint (step 1 & 2 above dotted line in Fig. 1), which includedFCM and cell sorting using antibodies against mutans streptococci. We mentioned advantages and demerit of this procedure. We also discuss on the plan of the further steps in this hypothesis.

II. MATERIALS AND METHODS

2.1 Strains Used In This Study And Culture Conditions

Laboratory strains, Streptococcusmutans C67-1, Streptococcus sobrinus 167, Streptococcus salivarius H26, Streptococcus mitis 9801 were cultured in brain heart infusion (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, disodium phosphate 2.5 g, per liter) (BD, Tokyo, Japan) with 1% sucrose (Sigma-Aldrich, Tokyo, Japan)at 37 °C in aerobic condition (Table 1). These strains were plated on mitis-salivarius (MS) agar (sucrose 50.0 g, trypan blue 75.0 mg, proteose peptone 10.0 g, crystal violet 0.8 mg,tryptose 10.0 g, potassium tellurite 1% 1.0 ml, dipotassium phosphate 4.0 g, agar 15.0 g, dextrose 1.0 g, per liter) (BD). To select mutans streptococci, MS agar were added 200U of bacitracin (Nacalaitesque, Kyoto, Japan) and 15% sucrose, 2mg of gramicidin D (Sigma-Aldrich), 10mg of colistin (Sigma-Aldrich), and 10mg of nalidixic acid (Sigma-Aldrich) per liter as Modified MS bacitoracin (MMSB) (28).

2.2 Antibodies Used In This Study

IgG to Strep. mutansdeveloped in rabbit by using Strep. mutansATCC25175 whole cell as immunogen, was purchased from Good Biotech Corp (GBC, Taichung, Taiwan). Goat anti-rabbit IgG conjugated with DYLIGHT® 488 (Excitation: 493nm, Emission: 518nm) was used as the secondary antibody (abcam, Tokyo, Japan). To reduce the background, human IgG (BD Fc Block reagent, BD, Tokyo, Japan) was used to block immunoglobulin-binding molecule of oral flora.

2.3 Saliva sampling

Human unstimulated saliva was obtained from volunteers, who gave informed consent. Saliva sampling was ethically permitted by Hiroshima University Ethical Research Committee (Epidemiology-No.1143). DMF (numbers of decayed, missing, and filled teeth) index was used as morbidity prevalence rate of dental caries. Sample A: a 52-yr-old male, DMF index: 10, sample B: a 22-yr-old female, DMF: 6, sample C: a 28-yr old female, DMF: 9, sample D: a 30-yr old female, DMF: 5, In the blueprint of this study, mothers' saliva are mandatory to use, however, saliva from adult volunteers including male and female, who were not caregivers, were used in preliminary experiments, in convenience.The volunteers had not taken any medication for 3 months prior to the study and did not use any chemical mouthwashes 3 hours prior to sampling. Eating and drinking were not permitted during the 2-h time period prior to sampling.If necessary, saliva was filtrated using 5 μm-pore filter (Merck Millipore, Tokyo, Japan)to remove desquamated epithelial cells.

2.4 Flowcytometry (FCM) andcell sorting

The sample preparation was as following.Bacteria or oral flora from fresh saliva were washed with PBS (pH7.4) (Nissui, Tokyo, Japan) twice by centrifugation at 12,000 rpm for 5 min using TOMY MX150 centrifuge and TMP25 angle rotor (TOMY SEIKO Tokyo Japan).Reaction of bacteria with 1st antibody was carried out by adding antibody with appropriate dilution. The reaction tube was incubated at room temperature for 15 min. Bacteria were washed with PBS (pH7.4) twice by centrifugation at the same condition as described above. Reaction of bacteria with fluorescence-labeled 2nd antibody was done at room temperature for 15min. Bacteria were washed with PBS (pH7.4) twice, followed by FCM analysis Flow cytometry used in this study was following. FACS Calibur (BD Biosciences, Tokyo, Japan) was used for the simple preliminary analysis of mutans streptococci living in saliva. The condition of FACS Calibur was following, Detector/Amp: P1 (FSC) E00, P2 (SSC) 300, P3 (FL1) 600, P4 (FL2) 550, P5 (FL3) 650, Threshold: all 0, no compensation. Ten data was acquired from 10,000 cells (bacteria).FACS Aria (BD Biosciences) was used for cell sorting to separateStrep. mutans from oral flora. The settings of FACS Aria is following; High frequency: 86.3, amplitude: 6.3, drop delay: 43.49, plate voltages: 6,000, voltage centering: 85, sheath pressure: 70. Cell sorting was performed by following the instructions provided.Sheath solution which is a liquid flow inside the lines of FCM, contain sodium azide at low concentration. As pilot test of plating bacteria on BHI agar plate after mixing sheath solution with Strep. mutans or saliva, it is confirmed that sheath solution did not affect the viability.

2.5 Quantitative real-time PCR

DNA extraction was performed using a DNeasy Blood and Tissue Kit for DNA extraction of Gram-positive bacteria (Qiagen, Austin, TX, USA). In general, DNA extraction from Gram-positive bacteria is more difficult than that from Gram-negative bacteria, because the cell wall peptidoglycan in Gram-positive bacteria is thicker than that in Gram-negative ones. Therefore, DNA extraction kit for Gram-positive bacteria was chosen. DNA concentrations were determined by measuring A260, and quality was estimated using the A260/A280 ratio (NanoPhotometer™ Pearl, Implen GmbH München Germany).For Quantitative real-time PCR (qPCR), Universal PCR Master Mix IIwere used following the instruction provided (Thermo Fisher Scientific, Yokohama, Japan). Amplification and detection were performed using an ABI PRISM 7700 sequence detection system (Thermo Fisher Scientific) with the following cycles: 50°C for 2 min, 95°C for 10 min, and 60 cycles of 95°C for 15 s and 58°C for 1 min (29).ΔRn represents the normalized reporter signal minus the baseline signal. The critical threshold cycle (CT) was defined as the cycle at which fluorescence became detectable above the background.The primers and probes used in this study were listed in Table 1.(targeted the gtfBgene of Strep. mutans, while another universal primer that targeted the eubacterial 16S rRNA gene was used to quantify the total bacterial load). Control DNA prepared from Strep. mutanswas used as following concentrations; 10^{-2}=10 ng, 10^{-3}=1 ng, 10^{-4}=100 pg, 10^{-5}=10 pg per each reaction. A standard curve was obtained from control PCR profiles. Then PCR profiles from F0-2 were overlaid on control profile curves. Calculation of bacterial numbers was carried out, based on the sensitivity as 1pg DNA = 12 cells (30, 31).

2.6 Microbiome

Prokaryotic 16S metagenomicssequencinglibrary was prepared using Illumina MiSeq System (Illumina, Tokyo, Japan). The primer pair sequences for V4 region created a single amplicon. These primers includes overhang adapter sequences for compatibility with Illumina index and sequencing adapters. Targeting this region are: 16S rDNA V4 PCR Forward Primer =
5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNGTGCCAGCMGCCCGCGGTAA-3'
[Sequencing primer: italic],16S rDNA V4 PCR Reverse Primer =
5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNGGACTACHVGGGTWTCTAAT-3'
[Sequencing primer: italic].

The Illumina overhang adapter sequences to be added to locus‐specific sequences are: Forward overhang: 5'-AATGATACGGCGACCACCGAGATCTACACTATAGCCTTCGTCGGCAGCGTC-3'‐[Index sequence: italic], Reverse overhang: 5'-CAAGCAGAAGACGGCATACGAGATTAGCGAGTGTCTCGTGGGCTCGG-3'‐[Index sequence: italic].

Sequencing was performed by MiSeq, using MiSeqv3 reagents. The MiSeqcan generate 70,980 reads for F0 sample and 27,000 for F2 sample. The Metagenomics analysis and taxonomic classification was carried out using MiSeqReporter.

2.7 Others: colony counts,bacterial-staining, microscopic observation

Bacterial colony counts were carried out by plating samples with several dilutions on BHI, MS, MMSB agar. Colony forming units (CFU) per ml was calculated by multiplying the dilution ratio. After FCM sorting, samples were arbitrarily diluted with sheath solution. In these cases, bacterial numbers were calculated based on the sample volume at saliva sampling.Experiments were triplicated to obtain averages and standard deviations.Heat-fixed smear cells spread on microscope glass slides were stained with crystal violet (Sigma-Aldrich) for 1 min.Cells were washed by indirect stream of tap water. After drying, microscopic observation was carried out under oil immersion at a magnification power of 100x and the 10x magnification of the ocular lens, using NIKON E1000M microscope (NIKON, Tokyo, Japan).A fluorescence microscope (NIKON E1000M) was used to observe fluorescence signals on mutansstreptpcocci reacted with antibodies at a magnification power of 1,000x. Fluorescence signals and phase contrast images were captured using CellSens Standard 1.11 software (OLYMPUS, Tokyo, Japan). Captured images were minimally processed using Powerpoint 2010. Brightness was adjusted to the entire images and all adjustment were within the linearity.

III. RESULTS

3.1 Optimization of antibody dilution

For the preliminary test, immunoreaction of 1strabbit IgG antibody to laboratory Strep. mutansC67-1 strain was carried out using 2nd goat anti-rabbit IgG conjugated with DYLIGHT® 488. After culturing Strep. mutans C67-1 for 2 days at 37 °C, immunoreaction and FCM analysis revealed that most of immunoreactedStrep. mutans(96.43 %) were found in UL region (Fig. 2A Data 1). Only 2nd antibody did not react to Strep. mutans, suggesting nonspecific reactions were rare in the case using laboratory Strep. mutans (Fig. 2A Data 2). When artificially mixed the sample of immunoreactedStrep. mutans (Data 1) and Strep. mutans without 2nd antibody reaction (Data 2), FCM analysis indicated clear separation between samples with or without 1st antibody (Fig. 2A Data 11). The optimal conditions were 100~1,000 times dilution of 1st antibody, and 10~100 times dilution of 2nd antibody.To know the positive rate of Strep. mutansC67-1 stained byimmunoreaction, fluorescence microscopic observation was performed (Fig. 2B). All Strep. mutans C67-1 showed fluorescence signals detected in all area observed, though there were cases where some signals were weak or not all chains of Strep. mutansC67-1 were stained (Fig. 2B arrow)

3.2 Specificity of 1st antibody

Since purchased 1st antibody to Strep. mutans was developed in rabbit by using Strep. mutans ATCC25175 whole cell as immunogen, it is possible to cross-react against other oral streptococci. To know the cross-reactivity of this antibody, several oral streptococci and Lactobacillus rhamnosusL8020were immunoreacted with 1st and 2nd antibodies. FCM analysis revealed strong cross reaction against Strep. sobrinus and Strep. salivarius, probably because several similar immunogens existed on the surface among these closely related bacteria (Fig. 3). Cross-reactivity to Strep. sobrinuswould be of benefit to the future separation of cariogenic bacteria from oral flora. However cross-reaction to Strep. salivarius was a little problem for this study. To solve this problem, it may be helpful to do a double staining with more than two antibodies recognizing different epitopes in Strep. mutans. The other way is to develop a new antibody that is more specific to Strep. mutans. Until then, we continue to use this antibody for next experiments, with an intention to contamination of Strep. salivarius at future FCM sorting. Because what we want to know is whether FCM sorting system can separate oral streptococci from oral flora. Therefore we decided to use this 1st antibody for the next experiments, even though cross-reaction was there.

3.3 Immunoreaction of antibody to oral flora

To see the immunoreaction of this antibody against oral flora, saliva from healthy volunteer (52-yr-old, male, DMF 10) was used,after checking the existence of Strep. mutans in his saliva by culture on modified mitis-salivarius bacitracin (MMSB) plate and PCR analysis. In experimental convenience we used saliva from MO, one of the authors, as a pilot study. FCM analysis of saliva using 1st and 2nd antibodies revealed an increase of population at upper left (Fig. 4, UL on Data 3). Only 2nd antibody reaction also showed a little increase of

population at upper left (UL on Data 5). However the reaction using both 1st and 2nd antibodies prevailed the population to that with only 2nd antibody. A little increase of population at upper left (UL) at the reaction using only 2nd antibody might occur because of the existence of nonspecific immunoglobulin binding molecule on the surface of several oral flora.Pretreatment of human IgG at 8 μg/ml reduced this nonspecific background at a certain degree (data not shown).Fluorescence microscopic observation of saliva stained with 1st& 2nd antibodies revealed many signals in which most of them correspond to Strep. mutans. A signal shown by arrow was big in size, being suggested to be a human cell detached from oral mucosa or garbage in the mouth. From these data, we decided to filtrate saliva in size of 5 μm, in which most of oral flora was able to go through but oral mucosa was not. We next attempted the same experiments using saliva from other three volunteers carrying mutans streptococci.Upper left population of Strep. mutansincreased in FCM analysis on saliva obtained from three volunteers (Fig. 5).These results suggested that mutans streptococci can be separated from oral flora, though this fraction somehow contained Strep. salivarius in some degree.

3.4 FCM and bacterial sorting

To recover oral flora without mutans streptococci, FACS Aria sorting system was used to separate mutans streptococci in saliva. Regions were surrounded as fluorescence-positive (F1) region and -negative (F2) region (Fig. 6A). Some spaces were intentionally set up between F1 and F2, because too close gate from F1 border to that of F2 makes trouble in sorting cells, especially cells located on the border between F1 and 2.Total 1,451,223 bacteria were analyzed and separated into fluorescence-positive (F1: 1,018,560) and -negative (F2: 432,663) fractions (Fig. 6A).Recovered each fraction was qualified by 2nd FCM analysis. Separation ratio was 84.3 % in F1 and 89.5 % in F2 after sorting. Quality and quantity analysis using culture technique with modified mitis-salivarius bacitracin (MMSB) plate revealed 31,300+/- 10,700 STD CFU/ml (15.5%: No. on MMSB / No. on BHI) were shown in F0, which was a fraction before FCMsorting. After cell sorting, 2,720 +/- 120 CFU/ml(3.32%) were grown in F1, 860 +/- 140 CFU/ml (0.89%)were in F2 (Fig. 7). Further quality PCR usingStrep. mutans primers and probe was performed. Calculated bacterial numbers were F0 = 15,635, F1 = 872, F2 = 94 cells, indicating that No. of mutans streptococci reduced in F2 fraction, although it was expected that F1 fraction had become more concentrated with Strep. mutans(Fig. 6& 7). We attempted to verify the separation of mutans streptococci from oral flora by crystal violet staining. Microscopic observation indicated that chain-shaped bacteria were rich in F1, on the other hands, microorganisms with sphere and short rod shapes were rich in F2. These results suggested the FCM & sorting can be strong tool to separate mutans streptococci from oral flora. However as we worried, many of Strep. salivarius might also be removed from the oral flora at the same time.

3.5 Microbiome analysis

FCM sorting is depend on the antibody used. In addition, several steps of FCM &sorting may expose oral flora to physical stress such as high fluidic pressure, laser beam, electrostatic charges, deflection through high voltage fields, and collisions with container surfaces (32). The numbers of general oral flora on BHI agar after FCM sorting were 97,040 +/- 54,160/ml in F2 fraction. We studied alteration of genera or species bymicrobiome of the oral flora before (F0) and after (F2) FCM and cell sorting. As shown in Fig. 8, the population ratio of streptococcus spp. decreased after FCM sorting. However population ratios of several species were also changed, especially in Neisseria, Agrobacterium and Fusobacterium spp. We expected to obtain almost same genera pattern before and after cell sorting, except Strep. mutans, Strep. sobrinus and Strep. salivarius but it did not. One of the reasons is a gate setting, which the border between F1 and F2 should be spaced. To solve this problem, we are planning to set the gate only F1 fraction (immunoreacted bacteria, mainly mutans streptococci) and to recover the other bacteria (no-immnunoreacted oral flora) from flow-through (from waste tank).

IV. DISCUSSION

Here, we proposed ahypothesis toestablish oral flora without mutans streptococci for dentalcaries prevention. To achieve this hypothesis, we attempted Step 1 and Step 2 of the blueprint in this study (Fig.1). Using rabbit IgG against Strep. mutans, FCM sorting system separated and recovered oral flora without mutans streptococci at high ratio, though there were a few problems. One was cross-reaction of 1st antibody to Strep. salivarius. Microbiome patternsalso showed changes on several strains before /after FCM sorting, even though Streptococcus spp. decreased after sorting.Before discussion on our results, we want to summarizerecent cariology and preventive dentistry of dental caries, again. Dental caries is multifactorial lesion (33, 34). There were transitions on theories from“specific and non-specific plaque theory” to “ecological plaque theory” or “keystone-pathogen hypothesis” (on mainly periodontitis) on development of oral diseases (35).Although many non-cariogenic bacteria were involved at several stages, final step to initiate dental caries was acidic environmental change, which thrived acidophilic bacteria,mutans streptococci and Lactobacilli(36).

Water fluoridation is recognized as an effective caries prevention method. However, in Japan, water fluoridation is banned by the government, probably because of adverse effects described in Introduction. Occurrence of dental caries in Japan are double compared with that of the United State (17). Our hypothesis and blueprint were originated from following four evidences. (i) In Japan, fluoridation is difficult, because of the government's decision. (ii) Oral flora including mutans streptococci are usually transferred from caregivers to their children. (iii) Mutans streptococci are established at so-called "window of infectivity" (24). (iv) It depends on person but ordinary parents do not like transplant of saliva to their own children from a complete stranger, even if he doesn't have any caries lesion at all.W proposed two solutions.

One is our presenting study on saliva transplant from caregivers to children, after removing mutans streptococci by FCM sorting. The other is to develop pills containing non-cariogenic oral flora based on the information obtained by analysis on non-cariogenic saliva. Culturing non-cariogenic oral, genetic study such as microbiome or biochemical information of saliva help to make artificial pills of non-cariogenic oral flora to transplant into babies' mouth. In fact, fecal transplant pills have already beendeveloped in the US (37). Recently fecal microbiota transplant becomes popular in the world. In general, fecal microbiota transplant is a transplantation of bacterial flora without Clostridiumdifficile from healthy donor to a recipient suffering from C.difficile infection. Careful selection of donors is important, especiallyon asymptomatic carriers of C.difficile. If FCM sorting system with antibody against C. difficile can remove it from fecal sample, it would be a good tool. Furthermore, self transplantation of fecal samples may be possible in some cases, after removing C. difficile or other pathogenic organisms from own fecal sample by this FCM sorting system. We also propose another procedure to reduce radiation stomatitis using FACS sorting. It is also self transplantation of individual saliva into their mouth. Patients with oral cancer frequently suffer from radiation- or chemotherapy-induced stomatitis, because the damage of oral tissues and a decrease of saliva flow. Our plan is following; So-called "healthy saliva" is pooled before radiation or chemotherapy. Since pooled saliva contains opportunistic pathogens, such as Candida albicans and/or Psuedomonas aeruginosa, these pathogens can be removed by FCM sorting using their antibodies. After removing pathogens, it is storedat -80 °C just like bacterial storage. When stomatitis begins, ice-form saliva is administrated into the mouth. It would help to recover their oral ecosystem, and to keep mouth moisture. In some cases, iced saliva is mixed with surface anesthesia ointment or fibroblast growth factor-related molecules to improve patients' oral conditions.

Our experiment performed in this study was on the way. Separation ratio between mutans streptococci and other oral flora was89.5 % (Fig. 6). It is also contaminated with S. salivalius to some degree.We need modification to improve separation ratio. Now we are preparing for specific antibodies to bind mutans streptococci. Not only single staining, but also double/triple staining of mutans streptococci using several antibodies will solve the problem on separation ratio. At cell sorting, setting of gate and recovery of oral flora from flow-through would improve separation ratio, too.Ifseparation ratios become better, oral flora withoutmutans streptococci will be administrated into the mouth at the period of "window of infectivity". Before that, animal challenge is mandatory to prove the establishment of artificial oral flora. To administrate the human oral flora without mutans streptococci into the mouth of baby mice or rats at the weaning periods, we have to separate baby mice or rats from their mothers. However, it may be difficult to separate baby mice or rats from their mother at the end of breast feeding, because bottle-feeding is almost impossible. In vitro caries model is one of another candidate to study on the effect of oral flora without mutans streptococci. According to the report on in vitro caries model, relatively-large amount of mutans streptococci were required to perform this experiment (38). However, there are few reports on in vitro caries model using oral flora complex (39). We have to think new methods to prove our hypothesis. Adult mice grown in asterile isolator, which carry no germ, will help us to prove the hypothesis, even though they are adult.

Oral flora itselfin saliva is not very harmful, because oral flora can spread any places by kissing, sharing tableware, sneeze and cough. However, oral flora after FCM sorting is a little skeptical about safety. Because the immunoreactions require antibodies and fluorophore. Sheath solution, a liquid flow inside the lines of FCM, sometimes contain sodium azide, an anti-septic agent. Several improvements are necessary for the further experiments. For example, antibodies should be developed from chicken or ostrich eggs. Because these eggs are edible. As a safe fluorophore, fortunately cells (bacteria) was recovered from non-fluorescent fraction by FCM sorting in this study, therefore, safety on fluorophore may not become a problem. If we are forced to say something on fluorophore, green fluorescent protein (GFP)-like molecule may be recommended, because some of jerry fish are edible.The other reason to use GFP is because GFP has been used as a tool to diagnose metastatic cancer inside the body (40). In addition of fluorophore, circular lines in FCM should be disposable for each person and sheath liquids should be sterile phosphate buffer or saline without azide.

H$_{ILLMAN}$ proposed replacement therapy of dental caries (17). They used Strep. mutansA2JMcarrying unique feature that an open reading frame of lactic acid dehydrogenase (LDH) gene was disrupted by inserting

alcohol dehydrogenase B gene, so thatStrep. mutansA2JM did not produce lactic acid.This strain naturally produced an antibiotics, mutacin 1140, which killed other strains of Strep. mutans (41). Combining Strep. mutans A2JM with amother's oral flora removed mutans streptococci by FCM sorting would be a better procedure for caries prevention in the near future. To do so, it is required to improve better separation ratio of mutans streptococci from oral flora by FCM sorting with not only single antibody but also double or triple staining, including anti-Strep. sobrinus antibody and/or anti-Lactobacillus. In the future, these improvements will help to establish FCM sorting system as a new method for prevention dental caries.

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LEGEND OF FIGURES

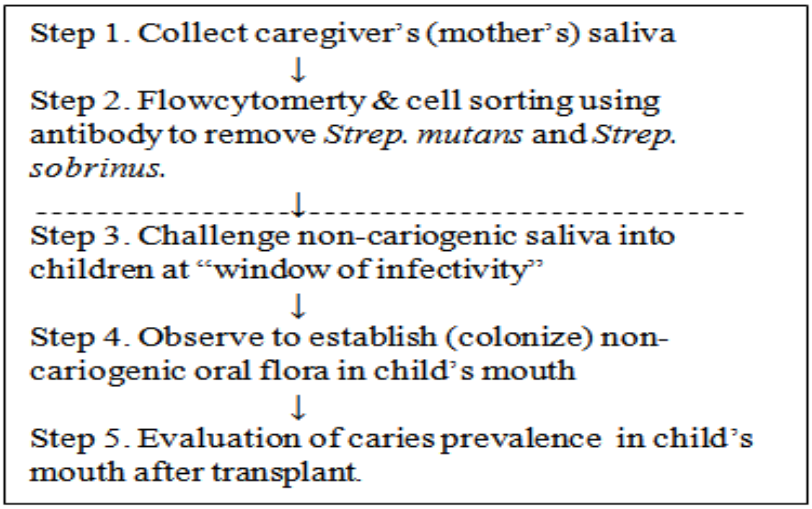
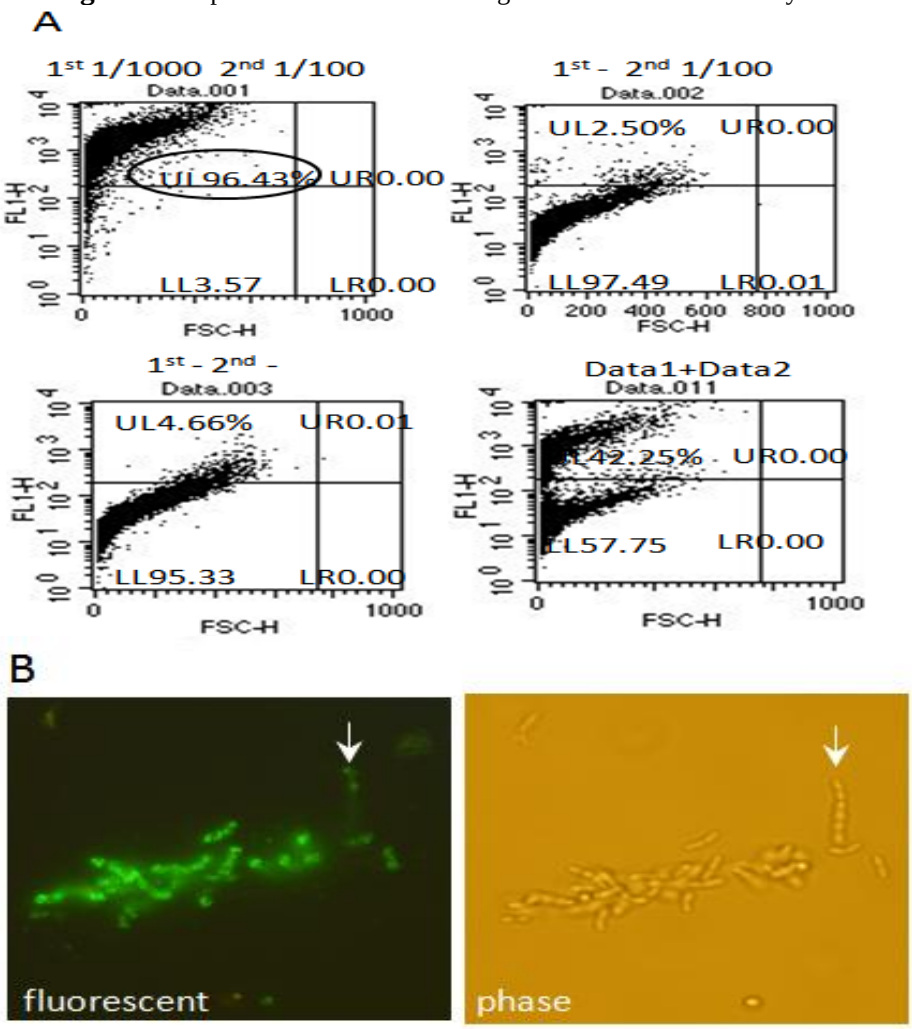


Fig. 1. A blueprint to establish non-cariogenic oral commensal ecosystem**.**



2. Immunodetection of Streptococcusmutans.

Laboratory Strep. mutansC67-1were immunoreacted with rabbit anti-Strep. mutans IgG as 1st antibody and 2nd antibody labeled with DYLIGHT-488. A: Flowcytometric analysis were performed at four conditions, Data1: 1st antibody (1/1,000 dilution) & 2nd antibody (1/100 dil), Data2: no 1st antibody & 2nd antibody (1/100 dil), Data3:

no 1st antibody & no 2nd antibody. Data1+Data2: Samples of Data1 and Data2 were artificially mixed and analyzed by FCM.B: Fluorescence microscopic observations on the sample from Data 1. All Strep. mutanswere positive, though some signals were weak or not all chains of Strep. mutanswere stained.

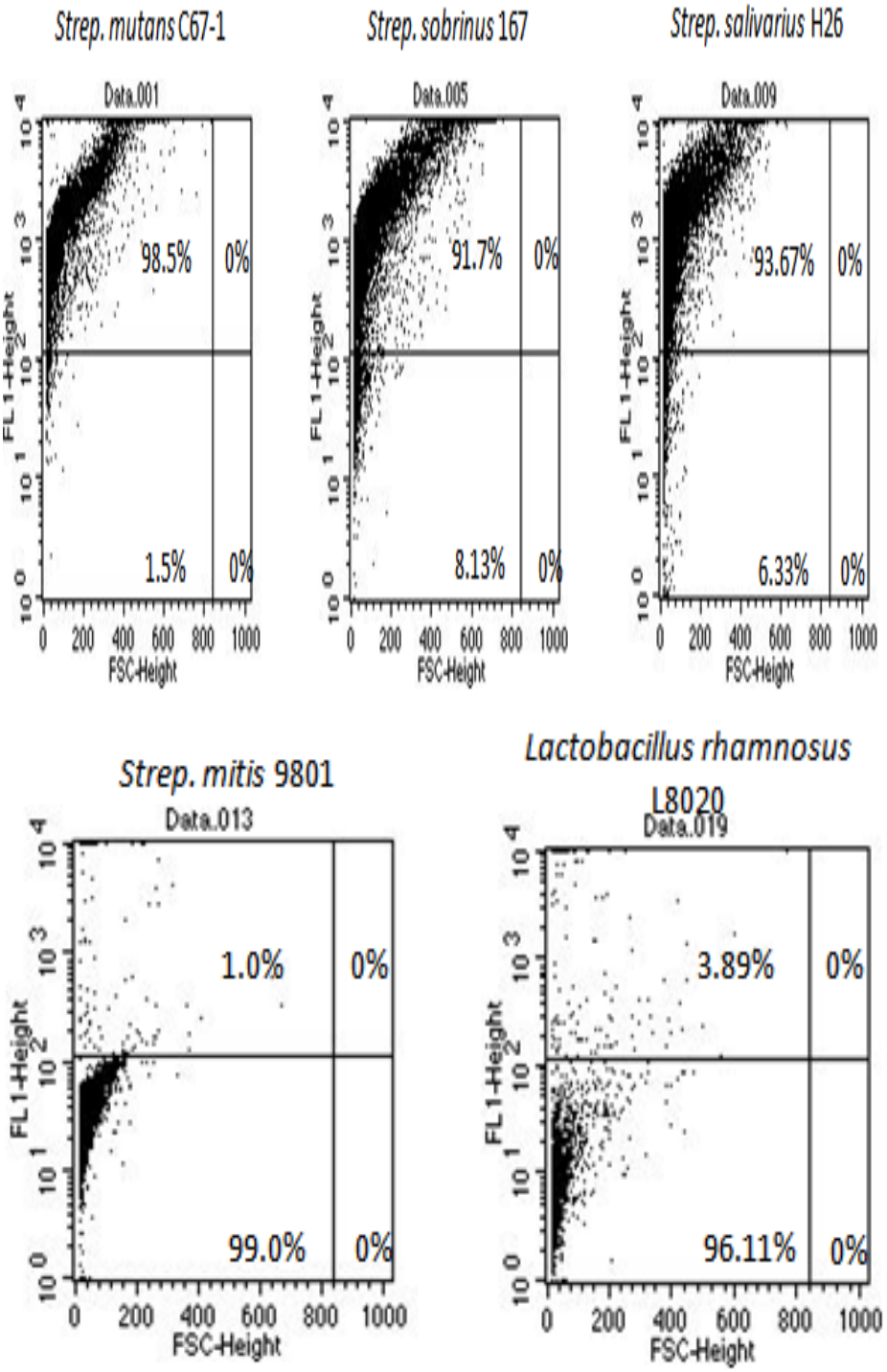


Fig.3. Cross-reactivity of 1st antibody against oral streptococci.

Several oral streptocci and Lactobacillus rhamnosuswere reacted with rabbit anti-Strep. mutans IgG used as 1st antibody (1/100 dil), followed by the reaction with DYLIGHT-488 labeled 2nd antibody (1/50 dil). Labeled cells were analyzed by FCM.

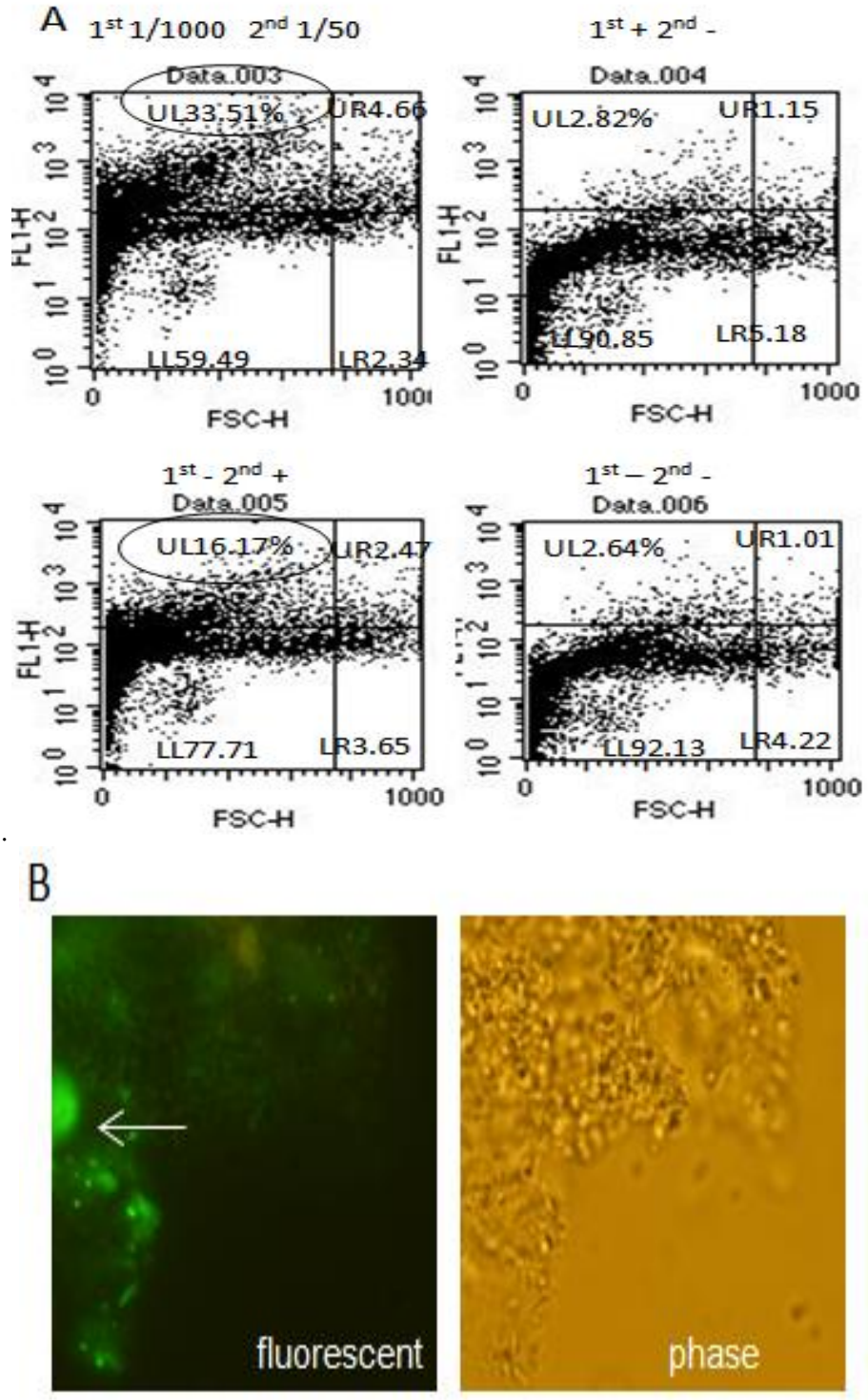


Fig.4. Immunodetection of Streptococcusmutansin human saliva.

Oral flora from a volunteer (sample 1: 52 yrs old, male, DMF: 10) was immunoreacted with rabbit anti-Strep. mutans IgG as 1st antibody and 2nd antibody labeled with DYLIGHT-488. A: Flowcytometric analysis were performed at four conditions, Data3: 1st antibody (1/1,000 dilution) & 2nd antibody (1/50 dil), Data4: 1st antibody (1/1,000) & no 2nd antibody, Data5: no 1st antibody & 2ndantibody (1/50). Data6: no 1st antibody & no 2nd antibody.B: Fluorescence microscopic observations on the sample form Data 3. Fluorescent signals of Strep. mutanswere detected among oral flora, including some background (arrow).

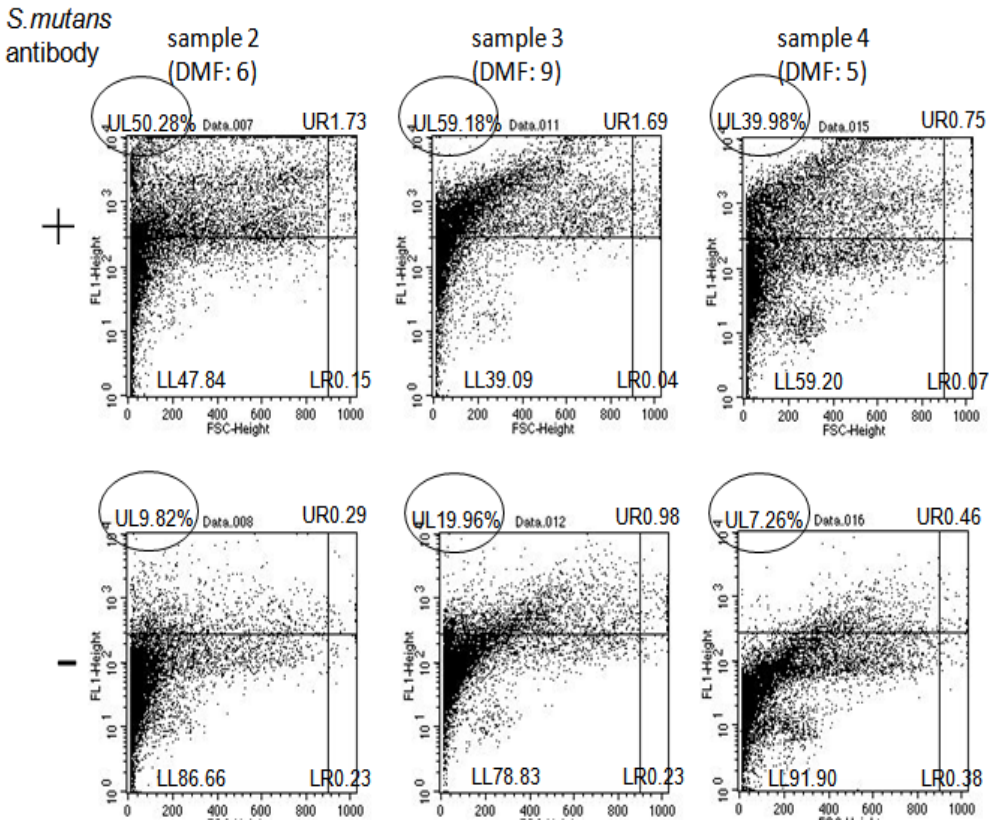


Fig.5. Immunodetection of Streptococcusmutansin human saliva from three volunteers.

Oral flora from 3 volunteers (Data 7,8: sample 2, 22 yrs old, female. Data 11, 12: sample3, 28 yrs old, female. Data 15, 16: sample4, 30 yrs old, female) were immunoreacted with rabbit anti-Strep. mutans IgG as 1st antibody and 2nd antibody labeled with DYLIGHT-488. Flowcytometric analysis were performed with/without 1st antibody, Data7, 11, 15: 1st antibody (1/1,000 dilution) & 2nd antibody (1/50 dil), Data8, 12, 16: no 1st antibody& 2nd antibody (1/50 dil).

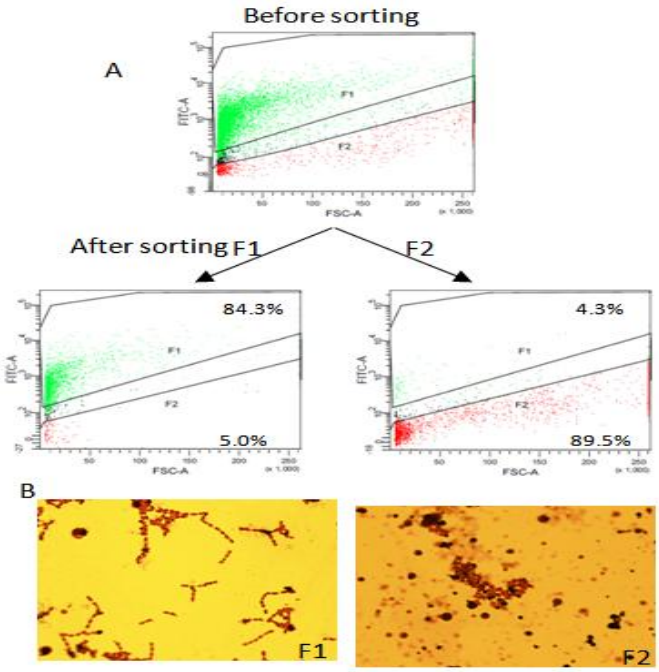


Fig.6. Cell sorting of oral flora by FACS Aria.

Cell sorting was carried out in saliva sample obtained from 52 yrs old, male shown in Fig.4, using anti-Strep. mutans IgG and DYLIGHT-488-labeled 2nd antibody. Upper panel of A is an FMC profile before cell sorting.Total 1,451,223 bacteria were analyzed and separated into fluorescence-positive (F1: 1,018,560) and -negative (F2: 432,663) fractions Cell numbers of 350,000 were in F2 fraction. A small portion (2,000 cells) from recovered samples were subjected flow cytometric analysis (A lower panel). Recovery ratio of each fraction was more than 80%. B: Microscopic observation of each fraction was done after crystal violet staining.

A

	BHI	MS	MMSB
F0	202,000±82,000	50,2000±338,000	31,300±10,700 (15.5%)
F1	74,480±37,520	71,920±28,880	2,720±120 (3.32%)
F2	97,040±54,160	58,880±16,320	860±140 (0.89%)

B

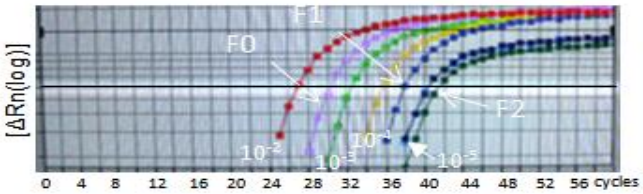


Fig. 7.Quantity of Strep. mutansafter separation by FCM sorting.

A: Bacterial numbers of F0 (before sorting), F1 (Strep. mutans-rich fraction), and F2 (Strep. mutans-poor fraction) were counted before/after FCM sorting as colony forming units using brain heart infusion (BHI) agar for general bacteria, mitis-salivarius (MS) agar for oral streptococci, and modified MS broth (MMSB) agar plates for mainly mutans streptococcus. B: qPCR to calculate cell numbers of each fraction after FACS sorting.Genomic DNA was prepared from each fraction, qPCR was carried out using ABI 7700 with Strep. mutans-specific primers and Fluorescence-labeled probe described in Materials & Methods. Standard curve, y = -1.954ln(x) + 50.356 was obtained from control Strep. mutansDNA sample (10^{-2}=10ng, 10^{-3}=1ng, 10^{-4}=100pg, 10^{-5}=10pg). Profiles of F0-2 were overlaid on control profiles. Calculated bacterial numbers were F0=15,635, F1=872, F2=94 cells, based on the sensitivity as 1pg DNA =12 cells (Oda 2015).

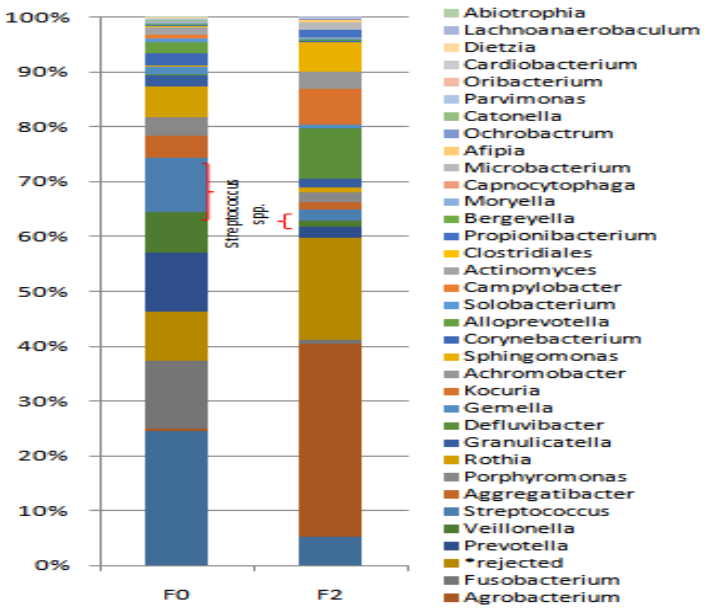


Fig.8. Microbiome before/after FCM sorting.

Comparison of species before and after FCM sorting was performed. The population (F2) of streptococcus spp. decreased after FCM sorting compared with those (F0) before sorting. However populations of several species were also changed.

antibodies		companies
1st antibody	Rabbit IgG against anti *Strep. mutans* ATCC25175 whole lysate	GBC
2nd antibody	Goat anti-rabbit IgG conjugated with DYLIGHT488 Ex: 493nm, Em: 518n	abcam
PCR primers & probes		
primers		amplicons
Smut3368f	5'-GCCTACAGCTCAGAGATGCTATTCT-3'	*gtf*B
Smut3481r	5'-GCCATACACCACTCATGAATTGA-3'	114 bp
Univ152f	5'-CGCTAGTAATCGTGGATCAGAATG-3'	16S rRNA
Univ220r	5'-TGTGACGGGGCGGTGTGTA-3'	69 bp
Fluorescent probes		
Smut3423T	5'-FAM-TGGAAAYGACGGTCGCCGTTATGAA-TAMRA-3'	*gtf*B
Univ117T	5'-FAM-CACGGTGAATACGTTCCCGGGC-TAMRA-3'	16S rRNA

Table 1. Strains, saliva, antibodies, and PCR oligonucleotids

	featurs	others
standards strains		
oral streptococci	*Streptococcus mutans* C67-1	
	Streptococcus sobrinus 167	
	Streptococcus salivarius H26	
	Streptococcus sanguinis 10556	
	Streptococcus mitis 9801	
Lactobacilli	*Lactobacillus rhamnosus* L8020	
		DMF
Saliva	sample 1: 52 years old male	10
	sample 2: 22 years old female	6
	sample 3: 28 years old female	9
	sample 4: 30 years old female	5
antibodies		companies
1st antibody	Rabbit IgG against anti *Strep. mutans* ATCC25175 whole lysate	GBC
2nd antibody	Goat anti-rabbit IgG conjugated with DYLIGHT488 Ex: 493nm, Em: 518n	abcam

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