



Long-term Effects of Eyewash Solution Use on the Conjunctival Bacterial Flora

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Abstract

Purpose: Commercial eyewash solutions are widely used in Japan for the removal of foreign bodies from the ocular surface and relief from unpleasant symptoms, and many individuals use them daily. However, the effects of habitual eye washing on the conjunctival bacterial flora have not been investigated yet. In this study, the bacterial flora in healthy eyes was evaluated after one month of continuous use of a commercial eyewash solution.

Methods: In total, 29 participants with healthy eyes were recruited. Eyebon® W, a commercial eyewash solution available in Japan, was used as the test eyewash. The eyes were randomly designated as treatment (group 1) and no treatment (group 2). The eyes in group 1 were washed twice daily with Eyebon® W for one month. The eyes in group 2 were not treated with any eyewashes or eye drops. Samples were collected from the inferior palpebral conjunctiva. Ultimately, a sufficient amount of cDNA for analysis was obtained from 11 individuals (22 eyes). The viable bacterial count was determined and the bacterial flora was analyzed using next-generation sequencing of 16S rRNA amplicons.

Results: In both groups, the bacterial flora was mainly composed of 6–7 genera. There were no statistically significant differences in the average rates for each genus in the conjunctival bacterial flora, regardless of the eyewash condition ($P > 0.05$).

Conclusion: Our findings suggest that washing eyes twice a day using eyewash is unlikely to have a significant impact on the conjunctival bacterial flora.

Keywords: Eyebon®; Eyewash; Ocular Surface; Microbial Flora; Safety; Long Time-use

Abbreviations

CFU: Colony-forming Unit; DEQS: Dry Eye-related Quality-of-Life Score; LASIK: Laser-assisted *in situ* Keratomileusis.

Introduction

Approximately 30% of the population in Japan has some sort of allergic predisposition [1]. This number has recently increased due to Asian dust, particulate matter PM2.5, and a rapid increase in the

number of patients with pollen allergies [2]. As a result, commercial eyewash solutions that remove foreign bodies from the ocular surface and provide relief from ocular discomfort are widely used in Japan, especially among contact lens wearers.

Eyebon® W (Kobayashi Pharmaceutical Co., Ltd.; Osaka, Japan) is a commercial eyewash solution that is designed to be used with a dedicated eyecup. This product has had the top share of the eyewash product market in Japan since its release in 1995. Eyebon® W not only washes away foreign matter from the ocular surface but also provides relief from the redness and itching caused by allergic conjunctivitis [3]. As a result, its use has become part of many consumers' daily routines. However, the habitual use of Eyebon® W initially proved problematic in terms of safety. In 2000, Watanabe, *et al.* reported that repeated eye washing with eyewash solutions containing a preservative (benzalkonium chloride) can cause disorders of the corneal epithelium [4]. After the publication of this report, Eyebon® W was redesigned to be free of preservatives. Although various reports have been published regarding its safety [5,6], to the best of our knowledge, few reports have investigated the association between eyewash solutions and eye infections. It, therefore, remains unclear whether eye washing using an eyecup can cause infections due to the flow of foreign matter into the eyes from the eyelids.

Eye infections are commonly caused by infectious keratitis which can be roughly classified into four different categories, namely bacterial, fungal, viral, and protozoal [7]. As most cases of fungal, viral, and protozoal infections are the result of extrinsic factors (e.g., wearing contact lenses and steroid use), eye washing is unlikely to cause these infections in healthy individuals. Although the resident conjunctival bacterial flora can be altered by various factors [8-10], there are currently no reports on the effects of eye washing on this bacterial flora. However, it is possible that eye washing could disturb the balance of the bacterial flora found in the conjunctival sac, which may then induce keratitis.

Hence, in this study, we investigated the effects of one-month use of a preservative-free eye washing solution on the resident conjunctival bacterial flora in healthy eyes.

Materials and Methods

Study participants and design

This study was performed in compliance with the Declaration of Helsinki of the World Medical Association and the ethical

guidelines for medical research conducted on human subjects. This study was approved by the Ethics Committee of Tsurumi University Dental Hospital and carried out between November 2017 and January 2018 in the form of a physician-led, multicenter, open-label, parallel two-group controlled trial. A series of research studies that included this study was registered as clinical trials at the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR registration number: UMIN000034516).

The number of participants was calculated using the following parameters, as previously reported [11]: error range (difference in terms of eye washing effect), 20%; confidence level, 95%; statistical power, 0.8%; standard deviation, 10. In terms of the effect of eye washing, a study population of eight participants was sufficient to detect significant differences ($P < 0.05$). Therefore, the target number of participants was set to 10. Participants had previously agreed to participate in research conducted at the testing institution (Central Research Laboratory, Kobayashi Pharmaceutical Co., Ltd.) and eye health was confirmed by prescreening. The exclusion criteria were as follows: Individuals with (1) a predisposition to an allergy to the constituents of Eyebon® W, (2) symptoms of allergic conjunctivitis (determined by the presence or absence of itching or hyperemia), (3) symptoms of dry eye (determined by the Dry Eye-Related Quality-of-Life Score [DEQS] and tear film break-up time), (4) keratoconjunctival epithelial disorders, (5) other ocular findings that might affect the safety or results of the study (i.e., glaucoma, blepharitis, iritis, pterygium, eye infections, or ophthalmic herpes zoster), (6) a history of vernal conjunctivitis or atopic keratoconjunctivitis, (7) ocular surgical procedures within the three months preceding the study or laser-assisted *in situ* keratomileusis (LASIK) surgery within the six months preceding the study, (8) uncontrollable systemic diseases, as well as individuals who (9) were pregnant, breastfeeding, or planning to become pregnant, (10) wore contact lenses one week before the study or during the study period, or (11) were taking medications during the study period. Individuals who met at least one of these criteria were excluded. All potential participants were screened on the same day. In total, 29 individuals who met the selection criteria (23 males, 6 females; mean age: 34.5 ± 7.3 years) were recruited as participants. All participants provided informed consent for eye washing and sampling. Eyebon® W (Kobayashi Pharmaceutical Co., Ltd.) was used as the test agent. The eyes to be tested were selected at random and washed twice a day continually for one month (group

1, N = 29). The remaining eyes were not treated with any eyewash or eye drops and were subjected to follow-up observation during the study period (group 2, N = 29).

The samples were evaluated by determining the viable bacterial count, followed by next-generation sequencing and 16S rRNA amplicon data analysis, as well as confirmation of the clinical findings. Intra- and inter-group comparisons were carried out at the beginning of the study and after one month. In terms of viable bacterial counts, specimens that did not yield any bacterial colonies were discarded. As a result, sufficient data were obtained for 10 eyes (group 1) and 9 eyes (group 2), from which colonies were obtained at both the beginning of the study and after one month. Similarly, during next-generation sequencing and 16S rRNA amplicon data analysis, any samples that did not yield sufficient amounts of cDNA for analysis were discarded. Ultimately, data were obtained from 22 eyes of 11 individuals for further analysis.

Test agent and eye washing method: Eyebon® W

Eyebon® W is a commercial eyewash solution sold in Japan. It is an over-the-counter drug that has been used in animal experiments and human clinical trials. Eyebon® W was designed and manufactured based on the criteria for the approval of the manufacturing and sales of over-the-counter drugs. Previous evidence has demonstrated that using Eyebon® W for eye washing for one month is unlikely to cause keratoconjunctival epithelial disorders, as well as having little effect on the mucin layer [5,6]. As of 2020, a total of seven types of Eyebon® eyewashes are sold on the market, all of which are preservative-free. In the present study, we used Eyebon® W, the most popular Eyebon® product. Eyebon® W is composed of the following active constituents (per 100 mL): dipotassium glycyrrhizinate (25 mg), chlorpheniramine maleate (3 mg), pyridoxine hydrochloride (vitamin B6; 10 mg), taurine (100 mg), cyanocobalamin (vitamin B12; 1 mg), and chondroitin sulfate (10 mg), as well as additives, namely boric acid, borax, polysorbate 80, sodium edetate, propylene glycol, l-menthol, dl-camphor, and a pH adjuster. Figure 1 shows the eye washing procedure in this study.

Conjunctival swab collection

Before sampling, 0.4% oxybuprocaine hydrochloride (Senju Pharmaceutical Co., Ltd., Osaka, Japan) was used as an anesthetic and applied to the eyes of the participants to reduce discomfort during sample collection. Next, the inferior palpebral conjunctiva was scraped using a sterile cotton swab (Nissui Pharmaceutical



Figure 1: Instructions for the use of Eyebon® W eyewash. (1) Pour the solution up to the line (5 mL) inside the attached eyewash cup. (2) Press the cup firmly to the eye and tilt the head backward, taking care not to spill the solution. (3) Wash the eye for 30 s by blinking several times. *Before administration, remove contact lenses, if worn, and wipe off cosmetics and blots around the eyes.

Co., Ltd., Tokyo, Japan). The sample was immediately dispersed in a sterilized normal saline solution in a 0.11 mL tube and stored at 4°C after which a solution of 0.3% sodium hyaluronate (Santen Pharmaceutical Co., Ltd., Osaka, Japan) was applied to the participants' eyes as a surface protectant.

Determination of the viable bacterial count

Within 24 h of sample collection, 0.06 mL of the collected samples were inoculated onto sheep blood agar medium (Nissui Pharmaceutical Co., Ltd.) and cultured at 35°C for two days, after which the number of colonies was determined.

16S rRNA amplicons and next-generation sequencing analysis

Using swab samples (80 µL of each), the V3-V4 hypervariable regions of 16S rRNA were amplified from the microbial genomic DNA by PCR using bacterial universal primers (341f/806r) and the dual-index method [12-15]. Barcoded 16S rRNA amplicons were sequenced using the paired-end, 2×284-bp cycle run on the MiSeq system with MiSeq Reagent Kit version 3 (600 cycles).

Statistical analysis

All statistical analyses were conducted using Excel 2013 for Windows (Microsoft Corporation, Redmond, WA, USA). Intra- and inter-group comparisons were performed using the Wilcoxon signed-rank test and the paired t-test, taking data variation into consideration. The results were analyzed using a significance level of 5%. Data are presented as adjusted means ± standard deviation. P-values less than 0.05 were considered statistically significant.

Results

Quantification of the number of viable bacteria

In group 1, the viable bacterial counts of the samples taken at the beginning of the study and those taken after one month were 465.0 ± 535.7 colony-forming units (CFU)/mL and 315.0 ± 373.3 CFU/mL, respectively. These values were not significantly different ($P > 0.05$). Similarly, the bacterial counts in group 2 were 894.4 ± 1146.6 CFU/mL and 1172.2 ± 1341.4 CFU/mL, respectively. These were also not significantly different ($P > 0.05$; Figure 2).

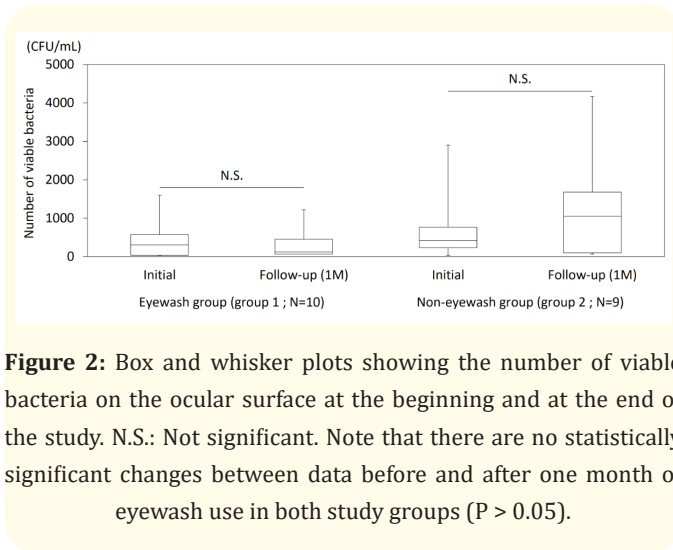


Figure 2: Box and whisker plots showing the number of viable bacteria on the ocular surface at the beginning and at the end of the study. N.S.: Not significant. Note that there are no statistically significant changes between data before and after one month of eyewash use in both study groups ($P > 0.05$).

Next-generation sequencing and 16S rRNA amplicon data analysis

Sequence analysis results revealed that an average of 23527.4 ± 6334.9 reads per sample was obtained. When a cutoff value of 3% was assigned to the average abundance ratio of each genus, the microbial flora in group 1 at the start of the study was composed of seven genera (*Brevundimonas*, *Pseudomonas*, *Staphylococcus*, *Delftia*, *Streptococcus*, *Corynebacterium*, and *Propionibacterium*). It should be noted that we classified bacterial genera with a mean abundance below 3% as “Others” and those that were unidentifiable as “Rejected hits.” The microbial flora present at the start of the study in group 2 had a similar composition (Figure 3A). In terms of the above-mentioned seven genera, whose initial abundance was 3% or higher, there were no significant changes in the mean abundance ratio for each genus one month after the begin-

ning of the study within either study group ($P > 0.05$ for all genera). However, the “Rejected hit” genera increased significantly in group 1, whereas the “Others” decreased significantly in group 2 (Figure 3B).

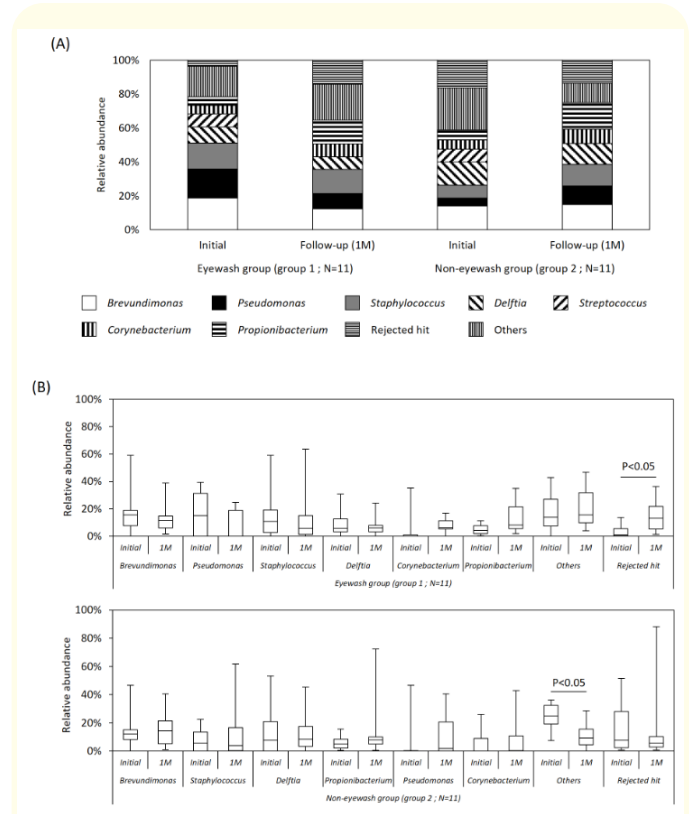


Figure 3: Relative abundance of each genus within the bacterial flora of the ocular surface. (A) Changes in composition and (B) comparison of relative abundance of each genus. Genera with a mean abundance below 3% are collectively indicated as “Others,” whereas those that were unidentifiable are indicated as “Rejected hits.” Note the nonsignificant changes in the relative abundance of the seven core genera before and after one month of eyewash use ($P > 0.05$).

Confirmation of Clinical Findings

Adverse events, such as dry eye or keratoconjunctival epithelial disorder, were absent throughout the study.

Discussion

Infectious keratitis, even in mild cases, is accompanied by hyperemia, tearing, and eye discharge, as well as ocular pain resulting in deterioration in the quality of life. In severe cases, this disease can induce corneal perforation and corneal opacity. In recent years, the widespread use of contact lenses has led to a rapid increase in the number of infectious keratitis cases [16]. The most common pathogens involved in infectious keratitis are bacteria, fungi, viruses (e.g., *Herpes* spp.), and protozoa (e.g., *Acanthamoeba* spp.). However, there are considerable differences among these pathogens. Bacterial keratitis is mainly caused by *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, which are also found in the residential bacterial flora of the eyelid skin and conjunctiva [17]. The same applies to fungal keratitis, whose main causative pathogens belong to *Candida* spp. and *Fusarium* spp., which are present on the eyelid skin and conjunctiva [18]. Herpetic keratitis is caused by the reactivation of latent herpes virus in the trigeminal ganglia, whereas acanthamoeba keratitis is caused by *Acanthamoeba* infections, which can be acquired from various sources, including tap water. In other words, among the four types of pathogens mentioned above, bacteria and fungi are commonly present in and around the eyes. As a result, the risk of infection is high, which is confirmed by the large number of cases observed in clinical settings. As such, eye washing using an eyecup, which allows for the washing of not only the ocular surface but also the eyelids, may result in the flow of bacteria and fungi into the eye and the disturbance of the bacterial flora. Furthermore, as commercial eyewashes are readily available to the general public, regulation of their use by physicians is not possible. This led us to conduct this study to investigate and evaluate the safety of eye washing using an eyecup.

The basic principle in the prevention and treatment of allergic diseases is the removal of the causative antigen. The same theory applies to ocular allergic diseases. As a result, the guidelines for the clinical management of allergic conjunctival diseases in Japan recommend self-care for antigen removal by various means [19]. However, eye washing is not recommended due to the risk of the flow of foreign matter from the eyelids into the eyes. This study focused on bacteria among the various foreign substances that could be encountered, and our results indicate that habitual eye

washing using an eyecup and a preservative-free eyewash slightly affected the resident conjunctival bacterial flora, although not to the extent that it produced adverse events. Furthermore, previous reports regarding eye washing demonstrate that using an eyecup and a preservative-free eyewash does not have any considerable impact on mucin (MUC5AC, MUC16) on the ocular surface [6] or cause any damage to the corneal epithelium, even after eye washing is performed six consecutive times [5]. These findings strongly support the safety of eye washing with a preservative-free eyewash using an eyecup.

The current study has several limitations. First, the evaluation was limited to bacteria. In theory, the study should also be conducted to assess the possibility that eye washing using an eyecup causes fungal keratitis. However, the incidence of fungal keratitis has been reported to be as low as 6–20% of that observed for bacterial keratitis, most cases being associated with steroid use [18]. Moreover, commercial eyewash solutions are not recommended for use in the case of inflammation, which requires steroid treatment [20]. Therefore, the idea of studying bacterial flora in the eyes of individuals with fungal infections was discarded. However, fungal infections cannot be ruled out among individuals who are taking oral steroids and washing their eyes using an eyecup. Therefore, an evaluation of eye washing in these individuals should be conducted in a future study. Second, quantification of the number of viable bacteria was limited to aerobic bacteria as the number of samples was limited. In theory, both aerobic and anaerobic bacteria should be quantified because they are present in the eyes. As a result, habitual eye washing over a period of one month was not accompanied by changes in the viable bacterial count. As the physical effects of eye washing using an eyecup are not believed to depend on bacterial species, a similar result would likely be obtained for anaerobic bacteria. Finally, individuals wearing contact lenses during the study period were excluded by our prescreening process. As mentioned previously, commercial eyewash solutions are used extensively by contact lens wearers, and the conjunctival bacterial flora in contact lens wearers is different from that in non-wearers. As contact lenses are typically used for several years instead of one month, as evaluated here), similar verification should be conducted over a longer period in future studies.

In this study, the main bacterial species detected belonged to seven genera, namely *Brevundimonas*, *Pseudomonas*, *Staphylococ-*

cus, *Delftia*, *Streptococcus*, *Corynebacterium*, and *Propionibacterium*. Several “Rejected hits” were also obtained. In a previous report, Dong, *et al.* [17] concluded that six of these genera (with the exception of *Delftia*) constitute the bacterial species that form the core of the resident conjunctival bacterial flora. As such, our findings are consistent with those by Dong, *et al.* Although *Delftia* was not part of the core of the resident conjunctival bacterial flora, many other genera were detected. Additionally, similar to previous reports [17,21], a certain number of “Rejected hits” was present in the resident conjunctival bacterial flora. Hence, further analysis is needed to determine which bacterial species are included in the resident conjunctival bacterial flora and whether there are differences among races. Furthermore, one month after the beginning of the study, there was a significant increase in “Rejected hits” in group 1 and a significant decrease in “Others” in group 2. However, the conjunctival bacterial flora composition varied among individuals, and previous reports have demonstrated the presence of unknown genera [17,21]. Moreover, adverse events were absent throughout the study. Therefore, we considered it unlikely that the resident conjunctival bacterial flora was significantly disturbed by

eye washing using an eyecup.

Conclusion

Our results indicate that the amount of residential bacterial flora present in the conjunctiva, as well as the composition of the microbiota, did not change significantly during the one-month period of daily eye washing using an eyecup. Eye washing offers various advantages and facilitates the prevention and management of eye diseases..

Acknowledgments

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Conflict of Interest

No competing financial interests exist. We also declare that a summary of this article was presented at the 30th Annual Meeting of the Japanese Society of Clinical Microbiology and the 45th Annual Meeting of the Society for Antibacterial and Antifungal Agents of Japan.

Supplementary Material

Sample_ID	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11
<i>Abiotrophia</i>	0	0	0	0	377	0	0	0	0	0	0
<i>Achromobacter</i>	96	0	715	475	0	0	0	0	0	0	0
<i>Acidaminococcus</i>	0	0	0	0	0	0	0	0	0	0	2
<i>Acidovorax</i>	0	0	0	0	0	1	0	0	0	1	0
<i>Acinetobacter</i>	0	1	1	1	0	0	0	0	0	0	0
<i>Actinobacillus</i>	0	0	0	0	0	0	0	0	0	2	1
<i>Actinomyces</i>	0	0	2	0	1	0	0	0	0	0	0
<i>Alicyclobacillus</i>	0	0	0	0	0	0	0	0	0	0	1
<i>Alistipes</i>	0	0	0	0	0	0	0	0	0	0	6
<i>Anaerococcus</i>	1399	0	0	1	0	0	0	0	0	0	0
<i>Anaerostipes</i>	0	0	0	0	0	0	0	0	0	0	1
<i>Anoxybacillus</i>	0	0	0	0	0	0	0	576	0	0	0
<i>Bacillus</i>	0	0	0	0	375	0	0	0	0	0	0
<i>Bacteroides</i>	0	0	0	1	0	0	0	0	0	0	15
<i>Bifidobacterium</i>	0	0	0	0	0	0	0	0	1	1	6
<i>Blastococcus</i>	0	0	0	0	0	1	0	0	0	0	0
<i>Blautia</i>	1	0	0	0	0	0	0	0	0	0	15

<i>Bradyrhizobium</i>	0	0	0	0	0	1	0	0	0	0	0
<i>Brevundimonas</i>	1268	4	1410	4028	5666	8717	2125	3961	3620	13403	1770
<i>Burkholderia</i>	0	0	0	0	1196	0	0	0	0	0	1925
<i>Capnocytophaga</i>	0	0	0	0	0	3661	0	0	0	0	0
<i>Chlorobium</i>	3	0	0	0	0	0	0	0	0	1	0
<i>Citrobacter</i>	0	0	0	0	0	0	0	0	0	1	0
<i>Cloacibacterium</i>	0	0	0	0	0	0	0	341	0	0	0
<i>Collinsella</i>	0	0	0	0	0	0	0	0	0	0	1
<i>Coprococcus</i>	0	0	0	0	0	0	0	0	0	0	1
<i>Corynebacterium</i>	5	1	2	7569	0	0	2841	497	0	1	0
<i>Delftia</i>	4106	5452	271	7	590	1148	4587	1349	1202	1045	1702
<i>Dermacoccus</i>	0	0	0	0	0	0	0	0	0	0	245
<i>Devosia</i>	0	0	0	0	1	0	0	0	0	0	0
<i>Dialister</i>	0	2	0	0	0	0	0	0	0	0	0
<i>Enterobacter</i>	0	0	0	0	0	0	0	0	0	0	4
<i>Escherichia</i>	0	0	0	0	0	0	0	0	0	0	15
<i>Eubacterium</i>	0	0	0	0	0	0	0	0	0	0	1
<i>Faecalibacterium</i>	0	0	0	0	4	0	0	0	0	0	6
<i>Finegoldia</i>	1	1096	48	0	0	0	0	1702	0	0	0
<i>Fusicatenibacter</i>	0	0	0	1	0	0	0	0	0	0	0
<i>Gemella</i>	0	0	0	0	0	0	0	0	0	0	1
<i>Granulicatella</i>	0	942	0	949	1	0	0	0	0	0	0
<i>Haemophilus</i>	0	0	0	2145	1	0	0	1	1	0	0
<i>Herbaspirillum</i>	0	0	0	0	0	0	0	0	0	0	1
<i>Kaistobacter</i>	0	0	0	1	0	0	0	0	0	0	0
<i>Lachnoclostridium</i>	1	0	0	0	0	0	0	0	0	0	0
<i>Lactobacillus</i>	0	0	0	0	0	0	0	0	0	0	1
<i>Lactococcus</i>	0	0	0	0	0	2	0	0	0	0	2
<i>Massilia</i>	0	0	655	0	0	0	0	0	3	0	0
<i>Megamonas</i>	0	0	0	0	0	0	0	0	0	0	1
<i>Methylobacterium</i>	0	0	187	0	0	0	1	0	6	0	0
<i>Methylophilus</i>	1	0	0	0	0	0	0	0	0	0	0
<i>Morganella</i>	0	0	0	0	0	0	1	0	0	0	0
<i>Mucilaginibacter</i>	0	0	0	1	0	0	0	0	0	0	0
<i>Murdochiella</i>	1	0	0	1	0	0	0	0	0	0	0
<i>Mycobacterium</i>	0	0	0	0	0	3697	0	0	0	0	0
<i>Neisseria</i>	0	0	313	0	1	0	799	0	0	0	0
<i>Not determined</i>	19	0	2200	2066	1	0	0	0	0	0	0
<i>Novosphingobium</i>	0	0	0	0	0	0	0	1	0	0	0

<i>Paenibacillus</i>	0	0	0	1	0	0	0	0	0	0	0
<i>Parabacteroides</i>	0	0	0	2	0	0	0	0	0	0	4
<i>Pedobacter</i>	0	0	0	0	0	1	0	0	0	0	0
<i>Peptoclostridium</i>	0	0	0	0	0	0	0	0	0	0	1
<i>Peptoniphilus</i>	2547	0	0	2	0	0	0	0	0	0	0
<i>Porphyromonas</i>	0	0	0	0	0	0	578	0	0	0	0
<i>Prevotella</i>	0	0	0	0	3	0	0	293	0	0	0
<i>Propionibacterium</i>	1721	1169	338	797	2983	2218	51	2367	241	460	943
<i>Pseudomonas</i>	2697	8880	3	9	13244	0	3864	4898	3132	0	8574
<i>Raoultella</i>	956	0	0	0	0	0	0	0	0	0	0
<i>Roseomonas</i>	0	0	0	1	0	0	0	0	0	0	0
<i>Rothia</i>	873	0	500	1	0	0	0	0	0	0	1
<i>Rubrivivax</i>	0	0	0	0	0	0	0	0	0	0	0
<i>Serratia</i>	97	0	634	0	1	0	0	0	331	0	0
<i>sp.Pseudomonas geniculata incertae_sedis</i>	3258	0	429	9	0	0	0	0	0	0	0
<i>Sphingomonas</i>	558	0	0	1	0	1834	0	0	0	0	0
<i>Sphingopyxis</i>	0	0	0	0	0	1	0	0	0	0	0
<i>Staphylococcus</i>	3827	1016	266	2570	11967	2	1	2257	12420	5086	2263
<i>Stenotrophomonas</i>	0	0	5	2	0	0	0	0	0	0	0
<i>Streptococcus</i>	0	377	11831	1	3	0	0	1067	1	2660	1255
<i>Subdoligranulum</i>	0	0	0	0	0	0	0	0	0	0	2
<i>Tyzzarella</i>	0	0	0	0	0	0	0	0	0	0	5
<i>Veillonella</i>	0	2299	0	0	0	0	0	1	0	0	0
<i>Rejected hit</i>	239	2493	470	857	80	163	26	1460	101	11	2997

Supplementary Table S1: DNA reads classification to the genus level in group 1 (initial).

Sample_ID	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11
<i>Achromobacter</i>	0	732	1570	0	0	0	0	0	0	0	0
<i>Acinetobacter</i>	0	0	0	0	1	1811	2	0	0	0	0
<i>Actinomyces</i>	0	0	0	0	0	550	0	0	1	0	0
<i>Alistipes</i>	0	0	0	0	0	0	0	0	0	0	1
<i>Anaerococcus</i>	0	0	0	0	0	0	0	18	0	0	261
<i>Anaerostipes</i>	0	0	0	0	0	0	0	0	0	0	0
<i>Bacteroides</i>	0	0	0	105	0	0	0	0	4	0	1
<i>Bifidobacterium</i>	2	0	0	0	0	0	0	0	4	0	0
<i>Blautia</i>	0	0	0	0	0	0	0	0	0	0	1
<i>Brevibacterium</i>	0	0	0	0	0	0	0	0	0	0	1
<i>Brevundimonas</i>	343	923	3225	1743	4800	2756	1768	988	10896	2350	3866

<i>Burkholderia</i>	0	0	0	0	0	0	0	0	0	0	743
<i>Cellulomonas</i>	0	0	0	0	0	0	0	0	0	0	170
<i>Chlorobium</i>	0	0	0	0	5469	0	0	0	0	0	0
<i>Citrobacter</i>	0	0	0	0	0	0	63	0	0	0	0
<i>Clostridium</i>	0	0	0	0	0	0	0	0	0	0	1
<i>Collinsella</i>	1	0	0	0	0	0	0	0	0	0	0
<i>Corynebacterium</i>	1130	2095	1572	1083	2	2959	3275	3486	3	1620	1271
<i>Delftia</i>	542	3	952	1110	1125	1463	2052	989	6760	3509	1422
<i>Desulfatirhabdium</i>	0	0	0	0	0	0	1	0	0	0	0
<i>Dialister</i>	0	0	0	0	1	0	0	0	0	0	0
<i>Enterococcus</i>	342	0	0	0	0	0	0	0	0	0	0
<i>Erysipelatoclostridium</i>	0	0	0	2	0	0	0	0	0	0	0
<i>Escherichia</i>	0	0	0	0	0	0	0	0	0	0	2
<i>Finegoldia</i>	903	0	0	595	0	1368	0	1521	0	0	0
<i>Fusobacterium</i>	0	682	0	0	0	0	0	0	0	0	0
<i>Gemella</i>	0	0	0	1	0	0	0	0	0	0	0
<i>Haemophilus</i>	0	1	0	0	1	0	0	638	0	0	0
<i>Hydrogenophilus</i>	0	0	0	1	0	0	0	0	0	0	0
<i>Intestinibacter</i>	0	0	0	0	0	0	0	0	0	0	1
<i>Lachnoclostridium</i>	0	0	0	0	0	0	0	0	0	0	1
<i>Lactobacillus</i>	0	249	0	0	0	0	0	0	0	0	0
<i>Lactococcus</i>	0	0	0	0	0	0	0	0	0	0	1
<i>Legionella</i>	0	0	0	0	0	0	0	189	0	0	0
<i>Mesorhizobium</i>	1087	0	0	0	0	0	0	0	0	0	0
<i>Methanospirillum</i>	0	1	0	0	0	0	0	0	0	0	0
<i>Methylobacterium</i>	0	269	0	0	0	0	263	1630	0	0	693
<i>Methylovorus</i>	0	0	0	0	0	0	0	0	1	0	0
<i>Microbacterium</i>	0	0	0	0	0	327	0	0	0	0	0
<i>Mobiluncus</i>	0	0	0	0	0	0	0	1113	1	0	0
<i>Moorella</i>	0	0	0	0	0	0	0	0	62	0	0
<i>Mucilaginibacter</i>	0	0	0	0	0	0	0	1	0	0	0
<i>Negativicoccus</i>	0	0	0	0	0	0	1	0	0	0	0
<i>Neisseria</i>	1	37	0	0	0	0	0	0	2	142	0
<i>Not determined</i>	4	3230	1	0	0	0	0	10	0	0	1
<i>Novosphingobium</i>	0	1342	0	0	0	0	0	898	0	0	0
<i>Paludibacter</i>	0	0	0	0	0	0	0	0	0	0	1
<i>Paracoccus</i>	0	0	0	0	0	0	0	0	0	164	0
<i>Pedomicrobium</i>	0	0	0	0	0	0	0	1	0	0	0
<i>Peptoniphilus</i>	0	0	1801	1	0	0	0	0	0	0	0

<i>Phascolarctobacterium</i>	0	0	0	0	0	0	0	0	0	0	1
<i>Porphyrobacter</i>	0	0	0	0	0	0	0	2	0	0	0
<i>Porphyromonas</i>	0	0	0	0	0	0	0	0	2008	0	0
<i>Prevotella</i>	0	0	243	0	0	0	0	1	3	0	0
<i>Propionibacterium</i>	1735	1389	1447	4695	6208	2841	9645	1815	7184	767	397
<i>Pseudomonas</i>	0	1	6012	4	7552	0	6	7883	1	3233	3077
<i>Raoultella</i>	609	1	0	0	0	0	0	0	0	0	0
<i>Rhizobium</i>	1	0	0	0	0	0	0	0	0	0	0
<i>Roseomonas</i>	0	0	3918	1	9	0	0	0	0	0	0
<i>Rothia</i>	0	0	0	0	0	1	1400	0	0	0	0
<i>Ruminococcus</i>	0	0	0	0	0	0	0	0	1	0	2
<i>Serratia</i>	0	806	3464	0	0	0	0	0	0	235	0
<i>sp.Pseudomonas geniculata incertae_sedis</i>	223	4	0	0	0	0	0	0	0	0	0
<i>Sphingomonas</i>	0	0	0	0	2	0	0	1	0	772	0
<i>Sphingopyxis</i>	0	0	0	0	0	1	0	0	0	0	0
<i>Sphingosinella</i>	0	0	0	0	0	0	0	1	0	0	0
<i>Staphylococcus</i>	13869	1785	2	2322	0	81	1614	752	741	3335	8070
<i>Stenotrophomonas</i>	0	8	0	0	0	0	0	25	0	0	0
<i>Streptococcus</i>	1	0	0	0	0	2248	2120	1255	2	326	0
<i>Syntrophomonas</i>	0	0	0	0	0	0	0	0	0	319	0
<i>Tepidimonas</i>	0	438	0	0	0	0	0	0	0	0	0
<i>Thermoleophilum</i>	0	0	0	0	0	0	0	0	1	0	0
<i>Thermus</i>	0	20	0	0	0	0	0	0	0	0	0
<i>Veillonella</i>	0	0	0	0	0	0	3760	0	0	0	0
Rejected hit	1011	2723	3686	6625	10129	1127	1596	8780	361	2705	382

Supplementary Table S2: DNA reads classification to the genus level in group 1 (after one month).

Sample_ID	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11
<i>Achromobacter</i>	1	0	1608	1715	0	608	0	0	0	0	0
<i>Acidaminococcus</i>	0	0	0	0	0	0	0	1	0	0	0
<i>Acinetobacter</i>	0	0	0	0	269	0	0	201	0	0	0
<i>Actinobacillus</i>	1	0	0	0	0	0	0	0	0	0	0
<i>Actinomyces</i>	992	2	0	3	0	1190	0	1135	0	0	0
<i>Akkermansia</i>	0	0	0	1	0	0	0	0	0	0	0
<i>Alistipes</i>	0	0	0	0	0	0	0	0	0	6	0
<i>Anaerococcus</i>	1	10	0	0	0	0	0	0	0	0	0
<i>Arenimonas</i>	0	0	0	0	0	1	0	0	0	0	0

<i>Bacteroides</i>	0	0	0	0	0	1	0	0	0	10	0
<i>Bifidobacterium</i>	0	0	1	0	0	0	0	0	2	2	1
<i>Blastocatella</i>	0	0	0	0	0	0	0	0	0	0	610
<i>Blautia</i>	0	0	0	551	0	0	0	1	0	7	0
<i>Bradyrhizobium</i>	0	0	0	0	0	0	0	1	0	0	0
<i>Brevundimonas</i>	751	5222	2419	2542	2838	5787	3555	9849	3	2336	2055
<i>Burkholderia</i>	0	0	0	0	0	0	0	0	0	0	2
<i>Campylobacter</i>	0	0	0	0	0	0	0	0	0	1	0
<i>Capnocytophaga</i>	0	1	0	0	0	0	0	0	0	0	0
<i>Cloacibacterium</i>	0	0	0	0	0	0	0	0	0	0	1
<i>Collinsella</i>	0	0	0	0	0	0	0	0	0	4	0
<i>Conexibacter</i>	0	0	0	0	0	0	1273	0	0	0	0
<i>Coprococcus</i>	0	0	0	0	0	0	0	0	0	3	0
<i>Corynebacterium</i>	3	1442	3102	4883	0	1	1265	1	2350	0	1
<i>Curtobacterium</i>	0	2573	0	0	0	0	0	0	0	0	0
<i>Delftia</i>	574	2421	4025	2	0	3660	1	60	6339	12736	8662
<i>Dorea</i>	0	0	0	0	0	0	0	0	0	0	1
<i>Enterobacter</i>	0	0	0	1	0	0	0	0	0	0	2
<i>Escherichia</i>	0	0	0	0	0	0	0	0	0	7	0
<i>Faecalibacterium</i>	0	0	0	0	0	0	0	0	0	4	4
<i>Fingoldia</i>	0	1408	0	0	0	0	0	791	0	0	0
<i>Fusicatenibacter</i>	0	0	0	1	0	0	0	0	0	6	0
<i>Fusobacterium</i>	0	0	0	0	0	864	0	0	0	0	0
<i>Gemella</i>	360	0	0	0	0	913	0	0	0	0	0
<i>Granulicatella</i>	0	0	0	0	0	782	0	0	0	1945	0
<i>Gulbenkiania</i>	0	0	0	1379	0	0	0	0	0	0	0
<i>Haemophilus</i>	753	0	0	3	0	2129	1549	0	0	1393	0
<i>Hymenobacter</i>	0	1282	0	0	0	0	0	0	0	0	0
<i>Hyphomicrobium</i>	0	0	0	1	1	0	0	0	0	0	0
<i>Intestinibacter</i>	0	0	0	0	0	0	0	0	0	3	0
<i>Kaistobacter</i>	0	0	0	5	1	0	0	1	0	0	0
<i>Kocuria</i>	0	0	0	0	0	0	0	0	0	0	5
<i>Lactobacillus</i>	0	0	0	0	0	0	0	0	0	1	0
<i>Massilia</i>	0	0	923	1	0	0	1	1	0	0	0
<i>Megamonas</i>	0	0	0	0	0	0	0	0	1	0	0
<i>Methylobacterium</i>	0	0	392	0	8	153	543	0	0	214	0
<i>Microbacterium</i>	0	0	0	0	0	1	0	0	441	0	1
<i>Moraxella</i>	0	0	0	0	1304	0	0	0	0	0	0
<i>Morococcus</i>	0	0	0	0	0	3	0	0	0	0	0

<i>Mycobacterium</i>	0	0	0	0	0	0	1424	0	0	0	0
<i>Nakamurella</i>	436	0	0	0	0	0	0	0	0	0	0
<i>Negativicoccus</i>	0	1166	0	0	0	0	0	0	0	0	0
<i>Neisseria</i>	789	0	0	982	0	5616	0	0	0	0	0
<i>Not determined</i>	791	0	1075	1	0	4	0	0	4326	1	0
<i>Olsenella</i>	1274	0	0	1	0	0	0	0	0	0	0
<i>Parabacteroides</i>	0	0	0	0	0	0	0	0	0	6	0
<i>Paucibacter</i>	12	0	0	0	0	0	0	0	0	0	0
<i>Pelomonas</i>	12	0	0	0	0	0	0	0	0	0	0
<i>Peptococcus</i>	0	0	0	0	0	787	0	0	0	0	0
<i>Peptostreptococcus</i>	879	0	0	1	0	0	0	0	0	0	0
<i>Phenylobacterium</i>	0	0	0	0	0	960	0	0	0	0	2637
<i>Planifilum</i>	0	0	0	0	0	0	0	0	0	0	3479
<i>Plasticicumulans</i>	0	0	0	0	0	0	0	0	0	0	1
<i>Prevotella</i>	0	748	0	0	0	0	1	0	0	8	0
<i>Propionibacterium</i>	313	3721	235	2933	813	1512	781	1141	1870	2057	325
<i>Pseudomonas</i>	1189	0	2	1	9907	1	1	2	3	1	3
<i>Raoultella</i>	0	0	0	1	0	0	0	0	0	0	0
<i>Roseomonas</i>	0	0	0	0	0	598	0	0	0	0	0
<i>Rothia</i>	0	0	1122	0	0	1247	732	1221	0	0	0
<i>sp.Pseudomonas geniculata incertae sedis</i>	0	0	3	0	0	0	0	0	4	0	0
<i>Sphingobium</i>	12	0	0	0	0	0	0	0	0	0	0
<i>Sphingomonas</i>	0	0	0	1	0	2	0	4295	0	2	576
<i>Staphylococcus</i>	5	1799	107	3162	272	10784	0	1668	2	2460	4392
<i>Stenotrophomonas</i>	0	0	0	0	0	0	0	0	5	0	0
<i>Streptacidiphilus</i>	0	0	0	1	0	0	0	0	0	0	0
<i>Streptococcus</i>	2030	2418	15971	9	3	6555	0	1	0	258	0
<i>Terrimonas</i>	356	0	0	1	0	0	0	0	0	0	0
<i>Thermoleophilum</i>	0	0	0	0	0	0	0	1	1	0	0
<i>Veillonella</i>	1	0	0	0	0	0	0	0	0	0	0
Rejected hit	12210	7214	571	532	5838	3658	4504	713	6369	363	220

Supplementary Table S3: DNA reads classification to the genus level in group 2 (initial).

Sample_ID	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11
<i>Achromobacter</i>	473	102	0	0	0	0	0	0	0	0	0
<i>Acidovorax</i>	0	0	0	0	1	0	0	0	1	0	0
<i>Acinetobacter</i>	0	286	544	0	0	32	0	6	0	0	0
<i>Actinomyces</i>	6	243	0	0	0	0	0	1	0	0	0

<i>Aeribacillus</i>	0	0	0	0	0	0	0	1	0	0	0
<i>Akkermansia</i>	0	0	0	0	0	0	0	0	0	6	0
<i>Alistipes</i>	0	0	0	0	0	0	0	0	0	8	0
<i>Anaerococcus</i>	2	0	29	0	1	0	0	0	0	0	0
<i>Anaerostipes</i>	1	0	0	0	0	0	0	0	0	0	0
<i>Anoxybacillus</i>	0	0	0	0	0	0	0	0	0	4	0
<i>Aquabacterium</i>	167	0	0	0	0	0	0	0	0	0	0
<i>Aquamicrobium</i>	172	0	0	0	0	0	0	0	0	0	0
<i>Arenimonas</i>	0	0	0	0	0	0	0	2	0	0	0
<i>Arsenicococcus</i>	1	0	0	0	0	0	0	0	0	0	0
<i>Bacillus</i>	3	0	0	0	741	0	0	0	0	0	0
<i>Bacteroides</i>	1	0	0	0	0	0	0	1	0	20	0
<i>Barnesiella</i>	0	0	0	0	0	0	0	0	0	2	0
<i>Bifidobacterium</i>	5	0	0	0	1	0	0	0	0	17	0
<i>Bilophila</i>	0	0	0	0	0	0	0	0	0	1	0
<i>Blastocatella</i>	0	0	0	0	0	19	0	0	0	0	0
<i>Blastococcus</i>	0	0	0	0	0	436	0	0	0	0	0
<i>Blautia</i>	0	0	0	0	0	0	0	0	0	12	0
<i>Bradyrhizobium</i>	11	0	0	0	0	0	0	0	0	0	0
<i>Brevibacterium</i>	1	0	0	0	0	0	0	0	0	0	0
<i>Brevundimonas</i>	299	1989	6134	2361	2538	146	8100	370	6059	5055	4106
<i>Burkholderia</i>	0	0	0	0	412	0	0	0	0	0	0
<i>Cellulomonas</i>	0	0	0	0	0	0	0	2	0	0	0
<i>Cloacibacterium</i>	2	0	0	0	2	0	0	0	0	0	1504
<i>Clostridium</i>	0	0	0	0	0	0	1	0	0	0	0
<i>Collinsella</i>	0	0	0	0	0	0	0	0	0	6	0
<i>Conexibacter</i>	0	0	0	0	0	1	0	0	0	0	0
<i>Corynebacterium</i>	1640	0	1023	7051	4101	0	2	0	64	4	4980
<i>Craurococcus</i>	0	0	0	0	13	0	0	0	0	0	0
<i>Delftia</i>	193	288	2303	2170	2529	2	1119	10129	5985	5419	1222
<i>Diaphorobacter</i>	43	0	0	0	611	0	0	0	0	0	0
<i>Duganella</i>	1	0	0	0	0	0	0	0	0	0	0
<i>Eggerthella</i>	0	0	0	0	0	0	0	0	0	1	0
<i>Eisenbergiella</i>	0	0	0	0	0	0	0	0	0	1	0
<i>Enterococcus</i>	0	0	0	0	0	0	0	0	0	2	0
<i>Enterorhabdus</i>	0	0	0	0	0	0	0	0	0	2	0
<i>Escherichia</i>	0	0	0	0	1030	0	0	0	0	10	0
<i>Eubacterium</i>	0	0	0	0	0	0	0	0	0	3	0

<i>Faecalibacterium</i>	0	0	0	0	0	0	0	0	0	10	0
<i>Finegoldia</i>	10	540	2714	0	396	0	0	0	1	0	0
<i>Fusicatenibacter</i>	1	0	0	0	0	0	0	0	0	3	0
<i>Fusobacterium</i>	1	0	0	0	0	0	0	0	0	0	0
<i>Granulicatella</i>	4	0	0	0	0	0	0	0	0	0	0
<i>Haemophilus</i>	1	0	0	0	0	0	0	0	0	0	0
<i>Hydrogenophilus</i>	0	0	0	0	2563	0	0	0	0	0	0
<i>Hymenobacter</i>	0	0	0	0	0	0	0	0	0	0	0
<i>Hyphomicrobium</i>	0	0	0	0	0	0	0	2	0	0	0
<i>Kaistobacter</i>	0	0	0	0	0	27	0	0	0	0	0
<i>Kocuria</i>	116	0	0	0	0	0	0	0	0	0	1
<i>Lachnoclostridium</i>	1	0	0	0	0	0	0	0	0	2	0
<i>Lactobacillus</i>	0	0	0	0	0	0	1	1	0	1	0
<i>Lactococcus</i>	0	0	0	0	0	0	0	0	0	1	0
<i>Massilia</i>	0	443	0	9	0	0	0	0	0	0	0
<i>Meiothermus</i>	0	0	0	0	0	0	0	2	0	0	0
<i>Methylobacterium</i>	1	0	11	17	0	0	782	767	604	1115	0
<i>Micrococcus</i>	0	0	0	0	1	0	0	0	0	0	0
<i>Modestobacter</i>	0	0	0	0	0	13	0	0	0	0	0
<i>Murdochiella</i>	0	0	0	0	0	0	2	0	0	0	0
<i>Mycobacterium</i>	378	0	0	1	0	0	0	1	0	0	0
<i>Negativicoccus</i>	122	0	0	0	0	0	0	0	0	0	1
<i>Neisseria</i>	43	0	0	0	1	0	0	0	0	0	0
<i>Nitrospira</i>	0	0	0	0	0	0	0	3	0	0	0
<i>Nocardioides</i>	0	0	0	0	1	0	0	0	0	0	0
<i>Not determined</i>	55	0	2798	0	7	24	0	0	0	8	0
<i>Odoribacter</i>	0	0	0	0	0	0	0	0	0	1	0
<i>Paludibacter</i>	5	0	0	0	0	0	0	0	0	0	0
<i>Parabacteroides</i>	0	0	0	0	0	0	0	0	0	8	0
<i>Peptoniphilus</i>	0	296	1	1	0	0	0	0	0	0	0
<i>Phascolarctobacterium</i>	0	0	0	0	0	0	0	0	0	5	0
<i>Plasticicumulans</i>	0	0	0	0	0	0	0	0	0	0	269
<i>Porphyromonas</i>	0	0	0	0	0	0	0	0	0	0	0
<i>Prevotella</i>	1	0	0	0	0	0	0	1	0	2	0
<i>Propionibacterium</i>	1693	16656	2300	1646	1774	75	244	10058	1889	935	2118
<i>Pseudomonas</i>	6	462	1	3	5832	1	8090	2	4982	7721	1155
<i>Raoultella</i>	282	0	0	1	0	0	0	0	0	0	0
<i>Reyranella</i>	3	0	0	0	0	0	0	0	0	0	0

<i>Roseateles</i>	1	0	0	0	0	0	0	0	0	0	0
<i>Roseomonas</i>	0	0	0	0	490	0	0	0	0	0	0
<i>Rothia</i>	148	0	261	0	0	0	0	0	0	0	0
<i>Rubellimicrobium</i>	0	0	0	0	2	253	0	0	1	0	0
<i>Ruminococcus</i>	0	0	0	0	0	0	0	0	0	4	0
<i>Saccharopolyspora</i>	0	0	0	0	88	0	0	0	0	0	0
<i>Serratia</i>	326	636	492	0	0	0	0	0	0	0	0
<i>Skermanella</i>	0	0	0	1	0	0	0	0	0	0	0
<i>sp.Pseudomonas geniculata incertae_sedis</i>	0	0	2	0	0	0	0	0	0	0	0
<i>Sphingobium</i>	0	0	0	0	0	0	0	3	0	0	0
<i>Sphingomonas</i>	7	326	0	0	2	375	0	6	0	0	1
<i>Staphylococcus</i>	14054	352	4287	2583	1150	76	0	0	1	4327	4504
<i>Stenotrophomonas</i>	0	0	8	0	0	0	0	0	0	0	0
<i>Streptococcus</i>	308	89	800	0	0	20	0	0	3727	0	0
<i>Thermoanaerobacter</i>	0	0	0	0	0	0	1	0	0	0	0
<i>Thermoleophilum</i>	0	0	0	0	0	0	0	0	0	0	9
<i>Tyzzarella</i>	0	0	0	0	0	0	0	0	0	2	0
<i>Variovorax</i>	0	0	0	0	5	0	0	0	0	0	0
<i>Veillonella</i>	92	0	0	0	0	0	0	0	0	2	0
Rejected hit	2046	291	3152	618	4112	11286	1564	887	497	201	1192

Supplementary Table S4: DNA reads classification to the genus level in group 2 (after one month).

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