Microbial interaction between human skin and *Nukadoko*, a fermented rice bran bed for pickling vegetables

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Article

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Abstract

The skin microbiome, which varies widely between individuals, plays a crucial role in human health. It also interacts with the environment in various ways, including during the preparation of fermented food. *Nukadoko*is a pickle and traditional fermented food in Japan that utilizes lactic acid bacteria to ferment vegetables. The microbial composition of *Nukadoko* contributes to the flavor of pickled products. However, the origin of the microbes present in the rice bran bed has yet to be determined and no studies have examined how the skin microbiome affects the quality of *Nukadoko*. In this study, the effects of interaction of microbes between *Nukadoko* and the human skin during *Nukadoko*maintenance were studied. Three participants were asked to stir commercially available late-stage *Nukadoko* for 14 days and not stir it for the remaining 14 days to examine microbial settlement and shedding. Microbiome analysis was performed on human skin and *Nukadoko*. We found that microorganisms from rice bran beds can temporarily settle on human skin but are shed quickly. Stirring rice bran beds by hand may have short-term effects on the skin microbiome. This study provides insights into the communication between human and food microbiomes in traditional Japanese fermented foods.

1. Introduction

Fermentation is a phenomenon used for food preservation carried out by microorganisms.
Among fermented foods, pickles, flavored by fermenting vegetables, are produced in food industries worldwide. Fermentation improves food preservation and aids in the development of its aroma, flavor, and texture. Lactic acid bacteria play a primary role in fermentation, specifically in the homofermentation and heterofermentation of lactic acid. Bacteria that are generally undesirable for food preservation, such as gram-positive bacteria, are vulnerable to low pH. These bacteria carry out fermentative production under anaerobic conditions to induce the growth of lactic acid bacteria and production of lactic acid (Voidarou *et al.* 2020). This type of fermentation is commonly observed in dairy products and in fermented vegetables (Ashaolu and Reale 2020).

Nukadoko, a traditional Japanese fermented food (Nakayama *et al.* 2007), is a rice bran bed that ripens pickles (*Nukazuke*). The traditional and predominantly manual method of preparing *Nukadoko* is to add salt water to the rice bran, knead it well, and then add vegetables to the rice bran bed for natural fermentation in the presence of lactic acid bacteria (Sakamoto *et al.* 2011; Ono *et al.* 2014). *Nukadoko* produced in this manner has a variety of microorganisms and contains a good balance of gram-positive bacteria, gram-negative bacteria, and yeast (Ono *et al.* 2014). This microbial composition imparts good flavor to the pickled products. Recently, the addition of fermentation starters, such as long-aged or commercially available *Nukadoko* which allows easier and more stable preparation and maintenance of the bed, has become the mainstream method (Sakamoto *et al.* 2011).

The microbial composition of *Nukadoko* has been investigated through massively parallel sequencing to identify 16S ribosomal RNA (16S rRNA) amplicon sequences (Nakayama *et al.* 2007; Sakamoto *et al.* 2011; Ono *et al.* 2014, 2015; Sawada *et al.* 2021). Pyrosequencing-based analysis revealed the microbial

dynamics of *Nukadoko* created in the laboratory with 16 different long-term aged bran beds as fermentation starters (Sakamoto *et al.* 2011). *Nukadoko* of different origins, in combination with fermentation starters, showed a variety of microbial compositions. Another study showed that the microbial diversity of *Nukadoko* with added spices, such as Japanese peppers and red peppers, differed because of the effect of secondary metabolites in spices (Ono *et al.* 2015). *Nukadoko* from different manufacturers has also been reported to contain different microbiomes (Ono *et al.* 2014; Sawada *et al.* 2021). Furthermore, diversity in organic and amino acids, which is influenced by microbiome variations (Sawada *et al.* 2021), significantly affects flavors.

However, for maintaining optimal microbial communities in *Nukadoko*, the rice bran bed requires stirring with bare hands either daily or every few days. The human skin is inhabited by various microorganisms that can affect fermentation (Byrd, Belkaid and Segre 2018). Previous studies have identified human skin-associated *Staphylococcus* in *Nukadoko* at an early stage of preparation (Ishizaki *et al.* 2001). However, no studies have examined how the skin microbiome, which varies widely from individual to individual, affects the quality of *Nukadoko*. Conversely, *Nukadoko* can contain microorganisms that may benefit the human skin. The effects of continued exposure to *Nukadoko* on the microbial composition of the human skin have never been thoroughly evaluated.

Using an interactive *Nukadoko* robot or Nukabot, we have previously investigated the emotional relationships between *Nukadoko* makers and its microbiome (Figure 1)(Chen *et al.* 2021). In the context of human–computer interaction, we evaluated the process of participants gaining awareness of native microorganisms through vocal conversation. When *Nukadoko* was stirred daily at increasing rates, more conversation took place, and a higher sense of emotional care was generated among the participants.

We used 16S rRNA amplicon sequencing to evaluate the effects of interaction between *Nukadoko* and the human skin microbiome during *Nukadoko* maintenance. Three anonymous participants maintained a commercially available *Nukadoko* at a late stage for 30 days. Shared amplicon sequencing variants (ASVs) were computed to identify microorganisms transmitted between *Nukadoko* and human skin. This study sheds light on the human–food microbiome interaction in traditional Japanese fermented foods.

2. Materials And Methods

Ethics

The study protocol was approved by the local ethics research committee of Waseda University (Ethics Review Procedures Concerning Research with Human Participants; application number: 2021-423; approved on February 7, 2022). All procedures were conducted according to the ethics committee's guidelines and regulations. All participants provided written informed consent before participating in the study.

Nukadoko maintenance and sample collection

The study participants were healthy volunteers recruited from acquaintances (N = 3); all were Japanese nationals, of which two were female, and one was male. The study was conducted in Tokyo, Japan, in February and March, 2022. The participants were given commercially available *Nukadoko* at the late stage and were asked to stir it for 14 days and not stir it for the remaining 14 days to examine microbial settlement and shedding on the skin. *Nukadoko* samples were collected on days 0, 3, 6, 9, 12, and 14 using individually wrapped disposable plastic spoons. Skin microbiome samples were collected on days 0, 3, 6, 9, 12, 14, 15, 18, 21, 24, 27, and 29 by swabbing the palm for 3 min using a sterile cotton-tipped swab (ESwab ™; Copan Diagnostics, Brescia, Italy). Swabs were stored in tubes with Liquid Amies Medium solution. (Copan Diagnostics, Brescia, Italy). Both sample types were immediately frozen and stored at -20°C until DNA extraction. The study workflow is illustrated in Figure 2. The sampling duration for each *Nukadoko* and skin microbiome sample was at least 6 h.

Total DNA extraction and high-throughput sequencing

Samples were treated with 750 µL of lysis buffer from the GenFind V2 DNA extraction kit (Beckman Coulter, Indianapolis, IN, USA). The suspension was vortexed for 10 min, heat-treated at 100°C for 10 min, and centrifuged for 5 min at 20000 g. The supernatant was then mixed with EZ beads (AMR, Tokyo, Japan), and DNA was fragmented using the MM-400 unit (Retsch, Haan, Germany) at a maximum speed for 3 min. The remaining DNA purification steps were performed using the abovementioned GenFind V2 DNA extraction kit (Beckman Coulter), according to the manufacturer's protocol. DNA was eluted with 80 µL of nuclease-free water; using the KAPA HiFi HotStart ReadyMix (Roche, Basel, Switzerland) (Caporaso *et al.* 2011; Klindworth *et al.* 2013) and specific primers (341F: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACACCTACGGGNGGCWGCA G-3') and 806R (5'-GTCTCGTGGGCTCGGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCT AATCC-3'), the V3–V4 region of the 16S rRNA gene was amplified. The thermal conditions were 95°C for 3 min, followed by 32 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. DNA samples, library preparation, and amplicon sequencing were performed using 300-bp paired-end sequencing on the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA) at GenomeRead Inc. (Kagawa, Japan).

Microbiome analysis

Microbiome analysis was performed as previously reported(Ito *et al.* 2022). Briefly, raw FASTQ files were imported into the QIIME2 platform (2022.8) as qza files(Bolyen *et al.* 2019). Denoising and read quality control were performed using the QIIME dada2 denoise-paired function, and reads were classified into ASVs (Callahan *et al.* 2016). We used 269 nt for-p-trunc-len-f and 255 nt for-p-trunc-len-r. The SILVA database's SSU 138 (https://www.arb-silva.de/documentation/release-138/) was used with the QIIME

feature-classifier classification scikit-learn package for taxonomic assignment (Quast *et al.* 2012; Bokulich *et al.* 2018). ASVs classified as chloroplast, mitochondria, or unassigned were excluded from subsequent statistical analysis. Subsampling is a common method for inferring microbiome differences between samples and is a suitable analytical approach for analyzing new datasets. To evaluate the effect of sequence read counts on microbiome diversity assessment, we plotted changes in the Shannon index over a range of read counts from 0 to 10,000, using rarefaction curves.

Custom database for taxonomic assignment

The classifier database used in this study was made from Silva release 138.1 SSU 99% (www.arb-silva) (Quast *et al.* 2012). Database curation was performed using REference Sequence annotation and CuRatIon Pipeline (RESCRIPt) following the developers' recommended parameters (Robeson *et al.* 2021). Briefly, RESCRIPt removed low-quality sequences (sequences containing >5 or more ambiguous bases or homopolymers of ≥8 bases) and filtered lengths (archaea [16S rRNA] ≥900 bp, bacteria [16S rRNA] ≥1200 bp, and eukaryotes [18S rRNA] ≥1400 bp). Additionally, deduplication of reads was performed in the Uniq mode. We then created the scikit-learn naive Bayes classifier using the QIIME2 plugin (feature classifier) (Bokulich *et al.* 2018).

Calculation of shared ASVs

We defined shared ASVs as ASVs shared by >1% of both datasets (*Nukadoko* and skin) in this study. When *Nukadoko* was touched for the first two weeks, data from *Nukadoko* and skin from the same day were used as pairs; when the bran was not touched for the next two weeks, data from *Nukadoko* from the last day and each skin microbiome data were used as pairs. The calculation was conducted using R (version 4.2.1) and phyloseq (version 1.40.0) (McMurdie and Holmes 2013) or the custom python code (q2-shared_asv v0.2.0, https://github.com/biota-inc/q2-shared_asv) with 0.01 for –p-percentage. Data were visualized using ggplot (version 3.4.0) and ggprism (version 1.40.0) (Wickham 2009; Dawson 2021).

3. Results

Nukadoko formed a conservative microbiome

First, 16S rRNA amplicon sequencing was performed to investigate the extent to which the skin microbiome affected the rice bran beds. After removal of mitochondrial and chloroplast-derived reads, we obtained 18,114 reads at maximum, 13,053 reads at minimum, and 15,937 reads at the median for *Nukadoko* samples and 21,706, 41,949, and 32,996 reads for skin samples. Details of the reads generated from DADA2 are presented in Supplementary table 1. We did not observe substantial changes in the microbiome composition of *Nukadoko* over two weeks compared with that on day 1. Specifically, the *Loigolactibacillus* genus was predominantly abundant among all three participants and accounted

for approximately 69–79% of the relative abundance throughout the 14 days (Figure 3A). *Pantoea* was the second most common genus, accounting for 5–10% of the total. *Xanthomonas* and *Staphylococcus* were also identified on all the days. However, the trend of *Loigonolactobacillus* comprising much of the microbiome composition did not change. Shannon diversity index, as an alpha diversity indicator, and other observed features did not show any substantial variation. The observed features were approximately 50, showing slight variation from day 0 to 14, and Shannon diversity index value was approximately 6, also showing daily and participant-specific variation, both slight (Figure 3B).

The skin microbiome varies from participant to participant

Participants stirred the bran and collected microbiomes from their palms using the swab method 6 h later by themselves. In contrast to the *Nukadoko* microbiome, the skin microbiome varied from participant to participant (Figure 4A). Across the participants, *Cutibacterium*, *Pseudomonas*, *Staphylococcus*, and *Acinetobacter* were the most common genera. *Acinetobacter* was more abundant in Participant 1, while *Cutibacterium* was more consistently identified in Participant 2, and *Kocuria* was particularly identified in Participant 3 than in the other two participants. Participants spent two weeks maintaining *Nukadoko* with monitoring and were further observed for two weeks without contact with it (Figure 4A, B).

The dominant genus in Nukadoko, *Loigolactibacillus*, was found in the skin microbiome of all study participants following their contact with *Nukadoko*, immediately after their interaction on Day 0, shown as Day 0’ (Figure 4A, B). To ascertain the degree of microorganism transfer between *Nukadoko* and the participants' skin, we calculated shared ASVs (Figure 4C). There were no shared ASVs detected prior to the act of stirring the *Nukadoko* on Day 0, with the only exception being Participant 3 (1.77%).

These shared ASVs originated from either *Loigolactibacillus, Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* group, or *Unassigned*, as shown in Figure 4C. Our observations confirmed that shared ASVs were present during the initial two weeks of stirring. In particular, we identified *Loigolactibacillus* accounted for over 10% of the shared ASVs on Day 3 in Participant 1, Day 3 and 6 in Participant 2, and Day 0’ and 9 in Participant 3. On Day 15, shared ASVs were found only on participants 1 and 3. After Day 15, we did not observe any additional instances of the shared ASVs.

4. Discussion

This study revealed that *Nukadoko,* at the late stage, formed an extremely conservative and stable microbial community. *Loigolactibacillus*, the dominant species of *Nukadoko*, was briefly transferred to the skin microbiome.

Out of the three stages of *Nukadoko*, the initial stage (before day 10), middle stage (day 10-30), and late stage (after day 30), the late stage was investigated in this study (Ono *et al.* 2014). Previous studies have

investigated the stable expansion of the microbiome in rice bran beds by inoculating plain rice bran with a fermentation starter and maintaining the transition of the microbiome through the three stages (Sakamoto *et al.* 2011). However, most customers buy commercially available matured *Nukadoko* and ferment vegetables by soaking them. No research has yet been conducted on microbiome variation during the maintenance of this fermented food at the late stage. To the best of our knowledge, this is the first study to address this issue. The most important characteristic of *Nukadoko* is that it requires careful stirring with bare hands by caretakers. Because of this, *Nukadoko* is always at risk of the easy introduction of foreign and undesirable microbes. The skin microbiome can also harbor bacteria that cause food poisoning, such as *Staphylococcus* (Kadariya, Smith and Thapaliya 2014).

In this study, three different participants maintained *Nukadoko* at home, and the microbiome hardly fluctuated in any of the batches over two weeks. The genus *Loigolactibacillus* was the priority species for the *Nukadoko* investigated (Figure 3A). Alternatively, *Nukadoko* with *Lactiplantibacillus plantarum* as the dominant species and extremely diverse microorganisms was previously reported (Ono *et al.* 2014; Sawada *et al.* 2021). One of the problems in this comparison is the reclassification of the *Lactobacillus* genus in 2020 (Zheng *et al.* 2020). A reanalysis of past studies is required to coordinate groups in *Nukadoko* based on their microbiome characteristics. Although absent in the sampled *Nukadoko*, *Halomonas* spp. has been reported to contribute to the elevation of glutamate concentrations (Sawada *et al.* 2021). Microorganisms in *Nukadoko* may contribute to the formation of flavors, and the role of each microorganism should be thoroughly investigated in future studies. *Nukadoko* is a fermented food that is customizable and requires consideration of numerous parameters to identify its chemistry, including the ingredients to be utilized, the location of the fermentation, and the people who will produce it. Thus, developing a microbiome database of the fermented food can lead to safer and more flavorful fermentation.

Several studies have used shared ASVs, including bacterial ASV transmission analysis, to determine the extent to which microorganisms are shared between mothers and infants (Maqsood *et al.* 2019) and a survey on microorganisms in milk collected from several regions and seasons in China (Liang *et al.* 2022). In our study, *Nukadoko* was collected before stirring, and skin samples were collected 6 h after stirring. Therefore, *Nukadoko* and skin samples were used for pairwise shared ASV analysis, allowing us to confirm the sharing rate on each day (Figure 4C).

16S rRNA amplicon sequencing is becoming an increasingly useful and affordable technique for microbiome screening. However, it has become clear that the results vary depending on the DNA extraction method, type of universal primer utilized, and method of analysis (Keisam *et al.* 2016). Similarly, some studies have reported that sampling methods also affect the alpha diversity of skin microbiome (Bjerre *et al.* 2019). Therefore, to allow for variations owing to technical problems, the threshold for shared ASVs was set to 1% in this study. Shared ASV is a valid calculation for identifying the microbial source of fermented foods but is limited by the shortcomings of 16S rRNA amplicon sequencing. To clarify the extent to which microorganisms have been transferred, it is necessary to detect cells at the single-cell level and comprehensively compare the results, using metagenomics. Another

technical limitation of 16S rRNA amplicon sequencing is the inability to distinguish between live and dead bacterial cells. To assess the impact of bacteria more accurately, it is necessary to employ culturing or staining-based methods that provide higher resolution. Also, 16S rRNA amplicon sequencing only detects bacteria, whereas yeast has been reported in rice bran. Yeast plays an important role in the flavor of bran as it is responsible for ethanol fermentation. It is necessary to investigate the amount of yeast present in bran beds by ITS amplicon sequencing or metagenomic analysis.

Studies of skin microbiome transfer have been reported in the past that considered the results of microbiome transfer from different donors to participants over a 24-h timescale (Perin, Addetia and Qin 2019). This study suggests that the microbiome implanted in the donor is present for 24 h. Our data are consistent with this, as *Loigolactibacillus* was identified on day 15, even after the participant stopped touching the bran on day 14. The microorganisms may have different effects on the host in terms of the settlement, but even touching the bran bed may cause attachment for a short period.

Another mystery in *Nukadoko* is that a substantial component of the rice bran microbiome can be human-derived microorganisms. Although there have been studies on the production of *Nukadoko*, there are no studies on the extent to which they are transferred from or contaminated with microorganisms of human origin at the initial stage of *Nukadoko*. In this study, we revealed a relationship between *Nukadoko* and the human skin microbiome, but microbial-level interaction between humans and *Nukadoko* remains unknown. Further research is required to identify host-food communication at the microbial level.

Declarations

Data Availability Statement

The datasets generated through 16S rRNA amplicon sequencing are available and deposited in the NCBI Sequence Read Archive (SRA) database under accession numbers DRR433234-DRR433293 and BioProject PRJDB14941.

Author Contributions

The study was conceived by all the authors. R.N., D.C. and K.I. designed the experiments. R.N. drafted the original manuscript. R.N. performed microbiome analysis. R.N. and K.I. performed statistical analyses. R.N. made q2-shared_asv. K.I. and D.C. collected samples. S.Y., K.J., and D.C. edited the manuscript and supervised the study. All authors have contributed to the manuscript and approved the submitted version.

Code availability

q2-shared_asv is available through https://github.com/biota-inc/q2-shared_asv.

Competing interests statement

K.I. is a board member at BIOTA Inc., Tokyo, Japan. R.N. is employed by BIOTA Inc. as a part-time developer. All other does not have any competing interest.

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Figures

Figure 1

Nukabot—an interactive *Nukadoko*robot (photo courtesy: Miraikan, The National Museum of Emerging Science and Innovation)

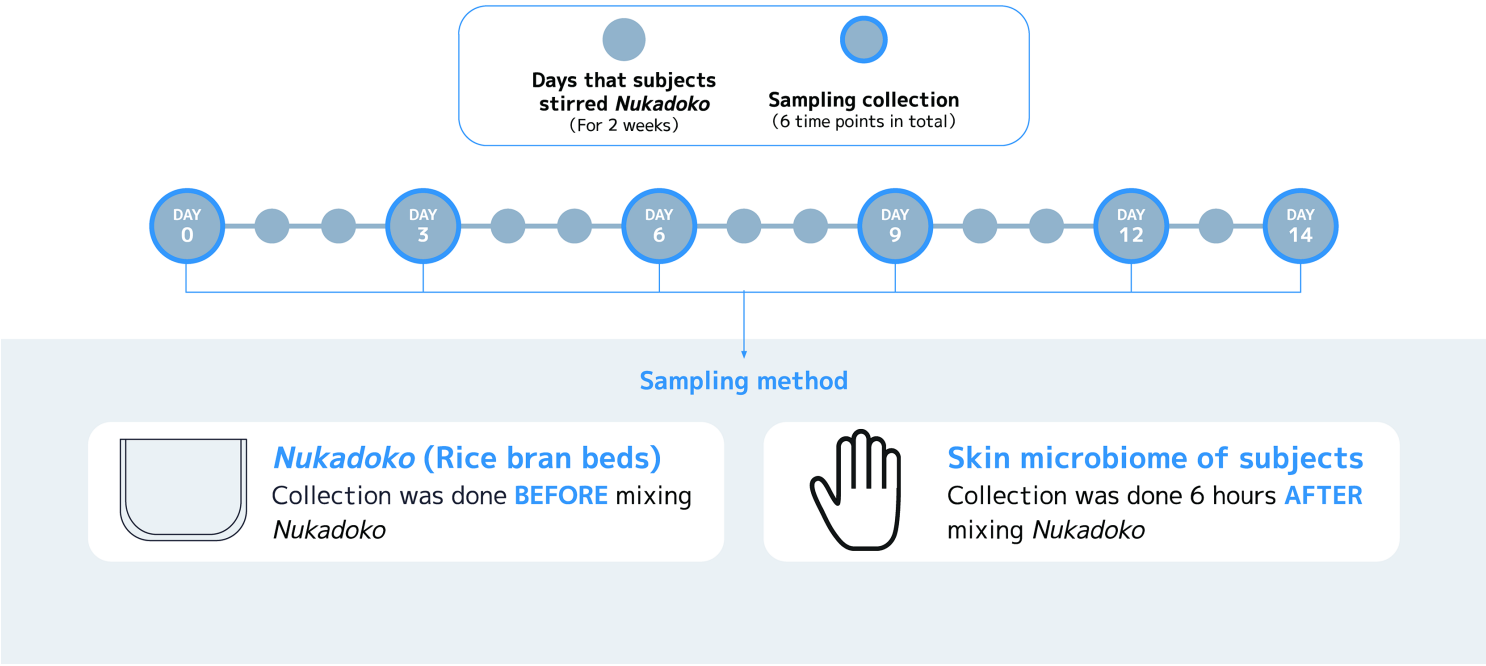

Figure 2

Study workflow. Monitoring was performed for two weeks, and *Nukadoko* and skin samples were collected as a pair.

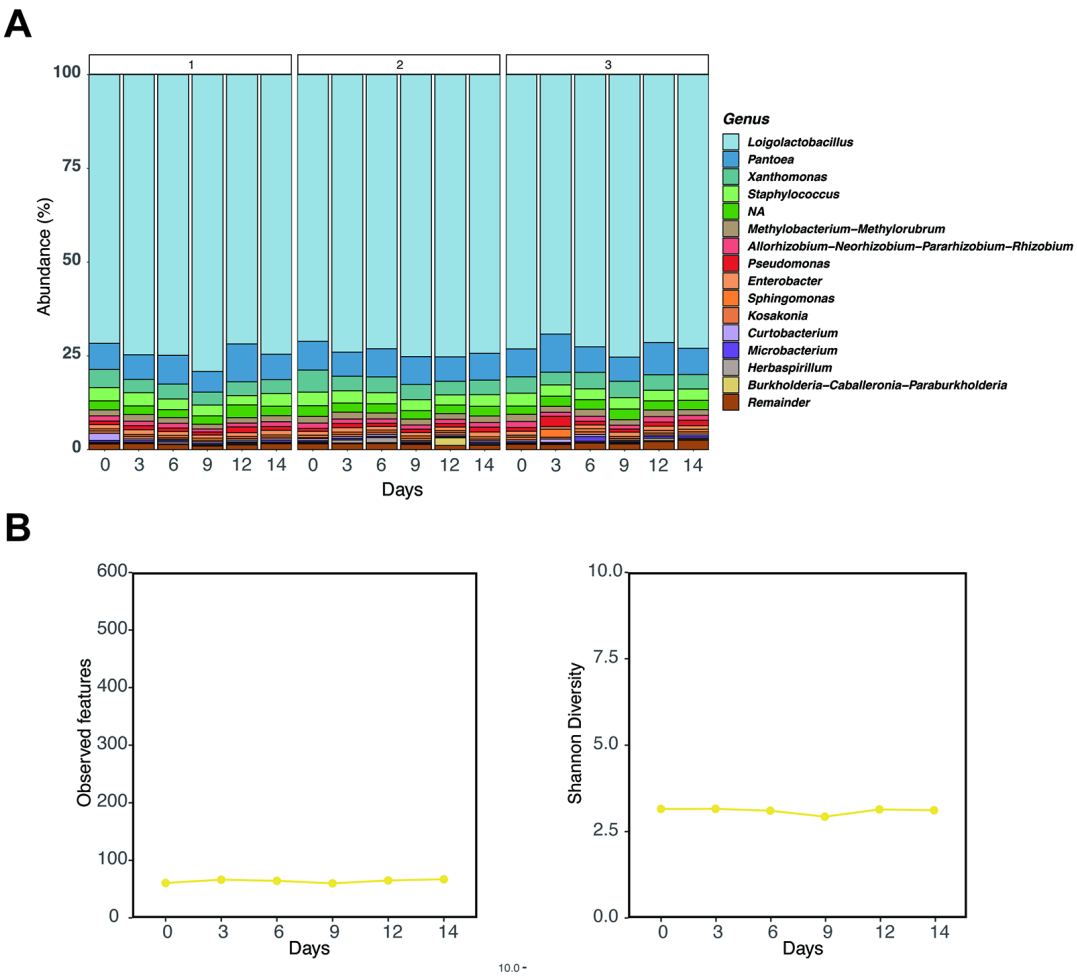

Figure 3

Changes in the microbial composition of the rice-bran beds. (A) Relative composition of the microbiome of rice-bran beds maintained by each participant over time. The top 15 genera are presented by their names, and the rest are grouped as the *remainder*. (B) Time-course changes in observed features and Shannon diversity index in microbial communities of rice-bran beds.

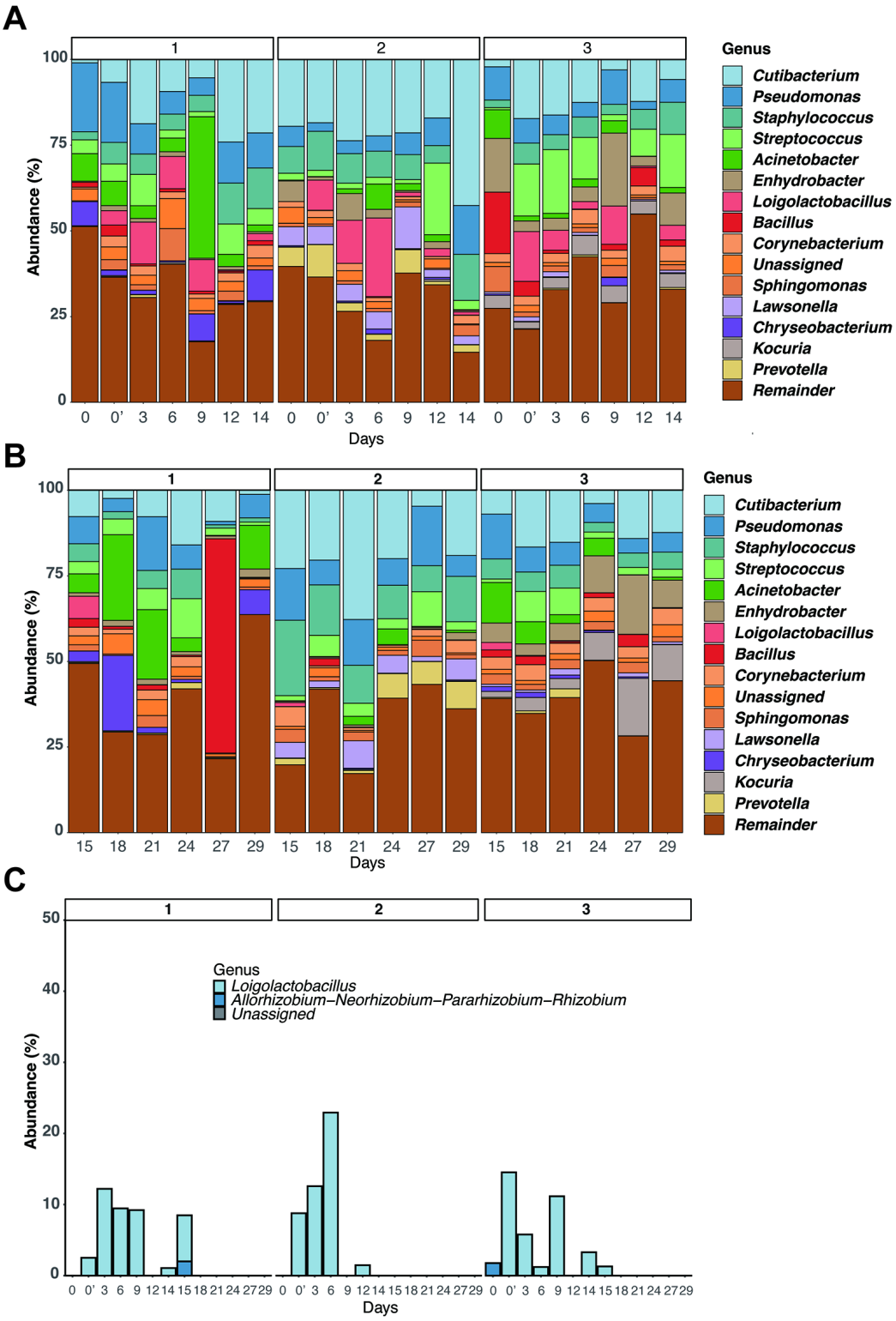

Figure 4

Changes in the microbial composition of the skin with the interaction of the rice-bran beds. (A) Relative composition of the skin microbiome of each participant over time from day 0 to day 14. The top 15 genera are presented by their names, and the rest are grouped as the *remainder*. (B) Relative composition of the skin microbiome without interaction with *Nukadoko* from each participant over time from day 15 to day 29. (C) The abundance of shared ASVs and their compositions.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementarytable.xlsx