Title

The similarities in microbial and chemical patterns of fermentation in two open environments were promoted by using 150-year-old *Nukadoko* as starters.

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Abstract

Background

Nukadoko, a fermented rice bran employed in traditional Japanese pickling, uses lactic acid bacteria to ferment vegetables. Here, we report the microbiome and chemical data of a mixture of matured 150-year-old *nukadoko* and commercially available rice bran placed in two open environments.

Results

Across the two environments, *Loigolactobacillus* was identified as the dominant microbial genera in the later stages of fermentation in *nukadoko*. The period of increase in the relative abundance of *Loigolactobacillus* correlated with a decrease in pH and ORP values. While the two environments showed a difference in the rate of change in microbial diversity, they shared the common process through which *Loigolactobacillus* outcompeted adventitious bacteria in *nukadoko*, as indicated by the alpha and beta diversity index.

Conclusion

Thus, the similarities in microbial and chemical data across two open environments during fermentation using starters indicate that starters contribute to the stability of fermentation in open environments.

Keywords

fermentation, microbiome, Nukadoko, lactic acid bacteria.

Introduction

Nukadoko, a fermented rice bran employed in traditional Japanese pickling, uses lactic acid bacteria (LAB) to ferment vegetables (*Nukazuke*). Traditionally, the *nukadoko* preparation process involves the natural fermentation of rice bran, continuously renewed by adding fresh bran. A previous study reported that the initial fermentation time was thereby reduced using an aged *nukadoko* as a fermentation starter ready for use (Ono *et al.*, 2014).

The microbiome of the fresh rice bran used in *nukadoko* is especially rich in microbial diversity, giving rise to the distinct flavor of *nukadoko* (Ono *et al.*, 2014). The type of rice bran used, salinity levels, temperature, air exposure, and fermentation time can be all examples of factors that distinguish the *nukadoko* microbiome (Osawa *et al.*, 2000). However, still little is known about the complete picture of the *nukadoko* microbiome, as well as the chemical changes during the fermentation process.

In this study, we examined the chemical and microbial changes during the fermentation of *nukadoko* in two different open environments—a public exhibition at Miraikan (The National Museum of Emerging Science and Innovation), and the Hakko Department (HakkoD), a food store specializing in fermented products, over 29 days.

Main Text

Materials and Methods

Preparation of *nukadoko* and sample collection

Nukadoko utilized in this study was prepared by mixing commercially available rice bran (Maruho Foods, Kagawa, Japan) with matured 150-year-old *nukadoko*. The long-aged (approximately 150 years) *nukadoko* used in this experiment were obtained from a *nukadoko* manufacturer (Hyaku-Gojunen-no-Nukadoko-Hozonkai) in Fukuoka City, Japan. The ingredients of the 150-year-old *nukadoko* were rice bran, salt, water, *kombu* (a type of edible seaweed), dried young sardines, bird's eye chili, Sichuan peppercorns, and rice malt. The mixed samples were placed in two locations for 29 days. 250 g of the 150-year-old *nukadoko* was mixed with fresh rice bran (500 g), salts (125 g), and distilled water (500 mL).

Three participants at both Miraikan and HakkoD were assigned to stir *nukadoko* on a daily basis at each location (Fig 1). The frequency of stirring was determined according to the hygiene standards of each facility and the operating patterns of the staff: once daily at HakkoD and twice daily at Miraikan, once every morning and once every evening. For each stirring session, the participants were asked to mix *nukadoko* thoroughly from the bottom 2-3 times, with each session spanning approximately 3 minutes.

Sample collection took place on specific days, namely 0, 3, 6, 9, 14, 18, 22, and 29 for Miraikan, and on days 0, 3, 6, 9, 15, 17, 22, and 29 for HakkoD using individually wrapped disposable plastic spoons and 50mL polypropylene conical tubes. Per samples were stored in a freezer after collection and kept at -20°C until DNA extraction after all samples were collected.

Chemical analysis

We employed high-precision pH and Oxidation-Reduction Potential (ORP) sensors for robots developed by Atlas Scientific (USA) and inserted them into the *nukadoko* rice bran. These sensors were connected to electronic circuits (Raspberry Pi 4) that transmit the sensed data to a cloud database (Amazon Web Service RDS) each minute during the whole period.

Total DNA extraction and high-throughput sequencing

DNA isolation from *nukadoko* samples was performed using the beat-beating method using the multi-beads shocker (Yasui Kikai, Osaka). The DNA was further purified using a GenCheck® DNA Extraction Kit Type S/F. The first PCR cycle was conducted using ExTaq HS DNA Polymerase, where specific primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') of the V3-V4 region of the 16S DNA were amplified. 2 ul of the first PCR product, purified using AMPure XP beads, served as a template for library preparation. The second PCR product was again purified using AMPure XP beads. Amplicon sequencing was performed using 460 bp paired-end sequencing on the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA) at FASMAC Co., Ltd (Kanagawa, Japan).

Microbiome analysis

Microbiome analysis was performed as previously regarding previous studies (Ito, Niwa, Yamagishi, *et al.*, 2023). Briefly, raw FASTQ data were imported into the QIIME2 platform (2022.8) as qza files (Bolyen *et al.*, 2019). Denoising sequences and quality control were performed using the QIIME dada2, and finally, sequences were produced into amplicon sequence variants (ASVs) (Callahan *et al.*, 2016). ASVs were assigned to the SILVA database's SSU 138 using the QIIME feature-classifier classification scikit-learn package (Quast *et al.*, 2013; Bokulich *et al.*, 2018). ASVs classified as mitochondria, chloroplast, or unassigned were excluded from subsequent analysis. Subsampling was performed to reduce bias due to differences in read depth between samples. The beta diversity indices weighted and unweighted UniFrac distances were calculated, and the microbial community structure differences between the two groups were visualized following a principal coordinate analysis (PCoA). Data were visualized using R (version 4.0.4), ggplot2 (version 3.4.3), and ggprism (version 1.0.4) (Creators Charlotte Dawson1 Show affiliations 1. University of Cambridge, no date; Wickham, no date).

Results

Changes in ORP and pH values during fermentation of *nukadoko*

Fig 2A shows the gradual decrease in pH values for both samples, indicating the fermentation rate. The most rapid decrease in pH occurred between days 3 and 7 for Miraikan and days 14 and 16 for HakkoD (Fig 2A).

The Oxidation Reduction Potential (ORP) showed variability throughout the experiment. The fermentation process leads to the consumption of dissolved oxygen by *Loigolactobacillus*, which produces lactic acid by breaking down carbohydrates (Wang *et al.*, 2021), resulting in lower ORP values. The decline in ORP values from approximately 200 to -100 occurs in the Miraikan sample across 8 days (days 0-7) and from approximately -50 to -250 occurs in the HakkoD sample across 5 days (days 9-13) (Fig 2B).

Increase the relative abundance of LAB

The ASVs from the samples were classified, with the most abundant genera being *Loigolactobacillus*, *Pantoea*, unknown sequences (NA), *Sphingomonas*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium, Pseudomonas*, and *Staphylococcus* on day 0 (Fig 3). Over time, *Loigolactobacillus* dominates the majority in both Miraikan and HakkoD samples. Despite the similar pattern, the rate of decrease in microbial diversity follows different paths, as the HakkoD sample continued to exhibit a wider variety of microbes over a longer period of time, whereas, in the Miraikan sample, *Loigolactobacillus* dominated the majority at a much earlier phase, at around day 6 of the experiment.

The microbial community structures of the two groups were compared using principal component analysis (PCoA) by Weighted Unifrac distance metrics. At the beginning, the two groups show a similar microbial composition, consisting of a broad range of microbial genera, as shown in Figure 2A. This is indicated by the close proximity with which the two sample points on day 0 are located on the PCoA plot. However, between days 3 and 9, the two groups exhibited distinct microbial compositions and eventually showed similarities again towards the end of the experiment (Fig 4A). Similarly to the results indicated by Figure 2A, the process of fermentation occurs over different periods. Indeed, at around day 3, the sample point from the Miraikan sample approaches the clustered sample groups, indicating the end of the fermentation process. However, in the HakkoD sample, the data points started to cluster together on day 14, displaying the gap in fermentation rate among the two sample groups. The sample points at the end of fermentation can be clustered together, where PC2 measures around 0%.

The Shannon Entropy values also exhibit similar results, where the decrease in the alpha diversity occurs exponentially, and then eventually stabilizes. The alpha diversity measures high among the HakkoD sample until day 14, when the graph merges with the Miraikan sample (Fig 4B). The decrease at the end coincides with the period *Loigolactobacillus* takes over as the most dominant genus. The days in which the alpha diversity decreases correlate with the days *Loioglactobacillus* dominates, occurring between days 6-7 for the Miraikan sample and days 14-15 for the HakkoD sample.

Discussion

Changes in ORP and pH values during fermentation of *nukadoko*

ORP values reading between -50 mV and -200 mV are considered micro-aerobic fermentation according to results obtained from ethanol fermentation using yeast (Liu *et al.*, 2016). The readings of ORP values obtained in this experiment mainly lie in the micro-aerobic fermentation level, indicating normal fermentation induced by the daily mixings and the concurrent air intake into *nukadoko*. This is also consistent with previous findings that ORP values reflect activities of aerobic (positive ORP) and anaerobic (negative ORP) bacteria, and thus ORP values are indicative of the level of fermentation (Chen, Ogura and Seong, 2019).

A previous study has also demonstrated the abundance of LAB and the accompanying pH decrease during the late fermentation period, similar to the results obtained in this experiment (Nakayama *et al.*, 2007).

Changes in taxonomic composition during fermentation of *nukadoko*

Our results showed that *Loigolactobacillus*, an anaerobic, homo-fermentative bacteria, became dominant in both environments. Similarly, in another study, *Loigolactobacillus* was identified as the dominant genus in *nukadoko* samples (Niwa, Chen *et al.*, 2023). The process through which adventitious bacteria are outcompeted by LAB at the later stages of fermentation is dependent on the types of LAB that dominate the microbiome (Ono *et al.*, 2014). A previous study (Ono *et al.*, 2014) investigated the chemical changes during the initial stage of fermentation across four samples made of different types of rice bran. Lactate concentration can be attributed to the differences between hetero-fermentative LAB during the early fermentation stage and homo-fermentative LAB at the later stages (Ono *et al.*, 2014). The dominance of *Loigolactobacillus* in our experiment suggests the abundance of homo-fermentative bacteria in 150-year-old *nukadoko*, indicating its matured fermentation state. It must be noted that *Loigolactobacillus* is a genus deriving from the reclassification of *Lactobacillus* (Zheng *et al.*, 2020).

In another study, Sakamoto identified two predominant species of *Lactobacillus* that maintain a *nukadoko* environment that prevents contamination by other microorganisms, conserving the distinct *nukadoko* flavor and preventing further spontaneous fermentation by adventitious microbes (Sakamoto *et al.*, 2011). Indeed, within the architectural environment in which the samples are placed, various microbes, known as the Microbiome of the Built Environment (MoBE), exist, which can influence the production of fermented foods (Gilbert and Stephens, 2018; Ito, 2023). MoBE has been found to contaminate the fermentation starter in a *sake* brewery (Ito, Niwa, Kobayashi, *et al.*, 2023). Similarly to the effects of *Lactobacillus* dominance of the microbiome in other experiments involving *nukadoko*, the presence of *Loigolactobacillus* in our experiment may also provide stability to the fermentation process and prevent adventitious microbes from contaminating the *nukadoko* environment. The high prevalence of *Loigolactobacillus* from day 0 may have resulted from an increased amount of readily matured *nukadoko*. We used an increased amount of starter compared to a previous study (Nakayama *et al.*, 2007) in order to support fermentation in open environments.

We also examined the microbial composition of *nukadoko* over the fermentation period, where most adventitious bacteria such as *Pantoea, Pseudomonas*, and *Staphylococcus* originating from sufficiently aged *nukadoko* were not detected by the end of the fermentation process in both Miraikan and HakkoD samples. Such changes in microbial composition resemble data obtained through previous studies, where previous study (Ono *et al.*, 2014) notes the changes from the prevalence of *Enterobacter*, a type of gram-negative bacteria encompassing *Pantoea*, to that of LAB in the later stages of fermentation. Thus, adventitious bacteria are continuously outcompeted by *Lactobacillus* and/or *Loigolactobacillus* and their lactic acid products. The LAB-dominant microbiome is not unique to *nukadoko* and can be seen in several fermented foods (Rezac *et al.*, 2018).

Figure 4A indicates that the two groups exhibit differences in the sample microbiome at the initial stages of fermentation but later show high similarities. Figure 4B indicates the gradual decrease in the Shannon Diversity index across two groups at the later stages of fermentation. It is suggested that these changes in microbial diversity reflect the gradual dominance of *Loigolactobacillus* throughout the fermentation process.

Limitation

Loigolactobacillus was detected at a relatively high abundance in the *nukadoko* from the initial stage of fermentation, which may have been caused by an excessive amount of starter. Due to the analysis at the ASV levels, it is yet to be known whether the detected *Loigolactobacillus* consists of a single or multiple species.

Ethics statement

The research protocol was approved by the Research Ethics Committee, Faculty of Design Engineering, Hosei University (Application Number:2022-4; approval date: March 28, 2023). All procedures were conducted according to the ethics committee's guidelines and regulations. Informed consent was obtained from all participants before starting this study.

Competing interests statement

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

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Figures

Figure 1. Maintenance of *nukadoko* in two open environments.

Figure 2. Time-series data of OPR (A) and pH (B) in Miraikan and HakkoD samples.

Figure 3. Genus-level taxonomic composition during the fermentation of *nukadoko* over 29 days. The top 15 genera are listed, and the rest are noted as Remainder.

Figure 4. Changes in the microbial diversity during the fermentation of *Nukadoko* samples. (A) Principal-coordinate analysis (PCoA) by Weighted Unifrac distance metrics of Miraikan and HakkoD samples. (B) Line plots of Shannon diversity index of Miraikan and HakkoD samples.

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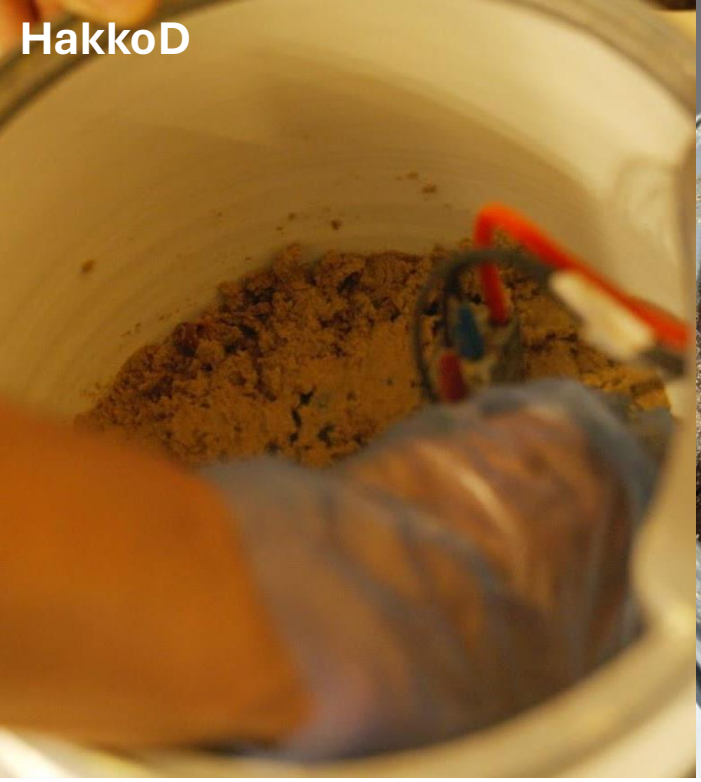
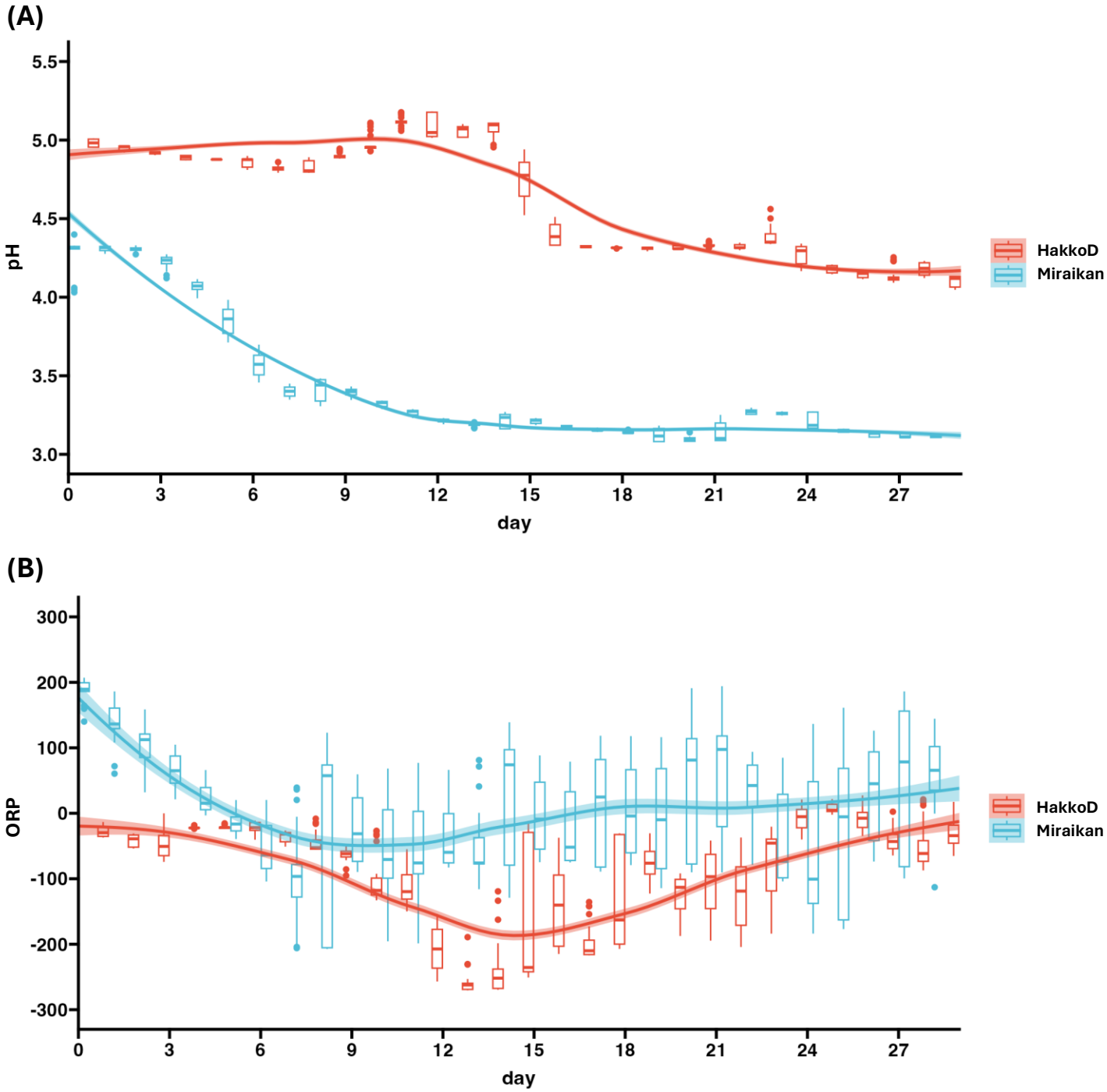

HakkoD

Miraikan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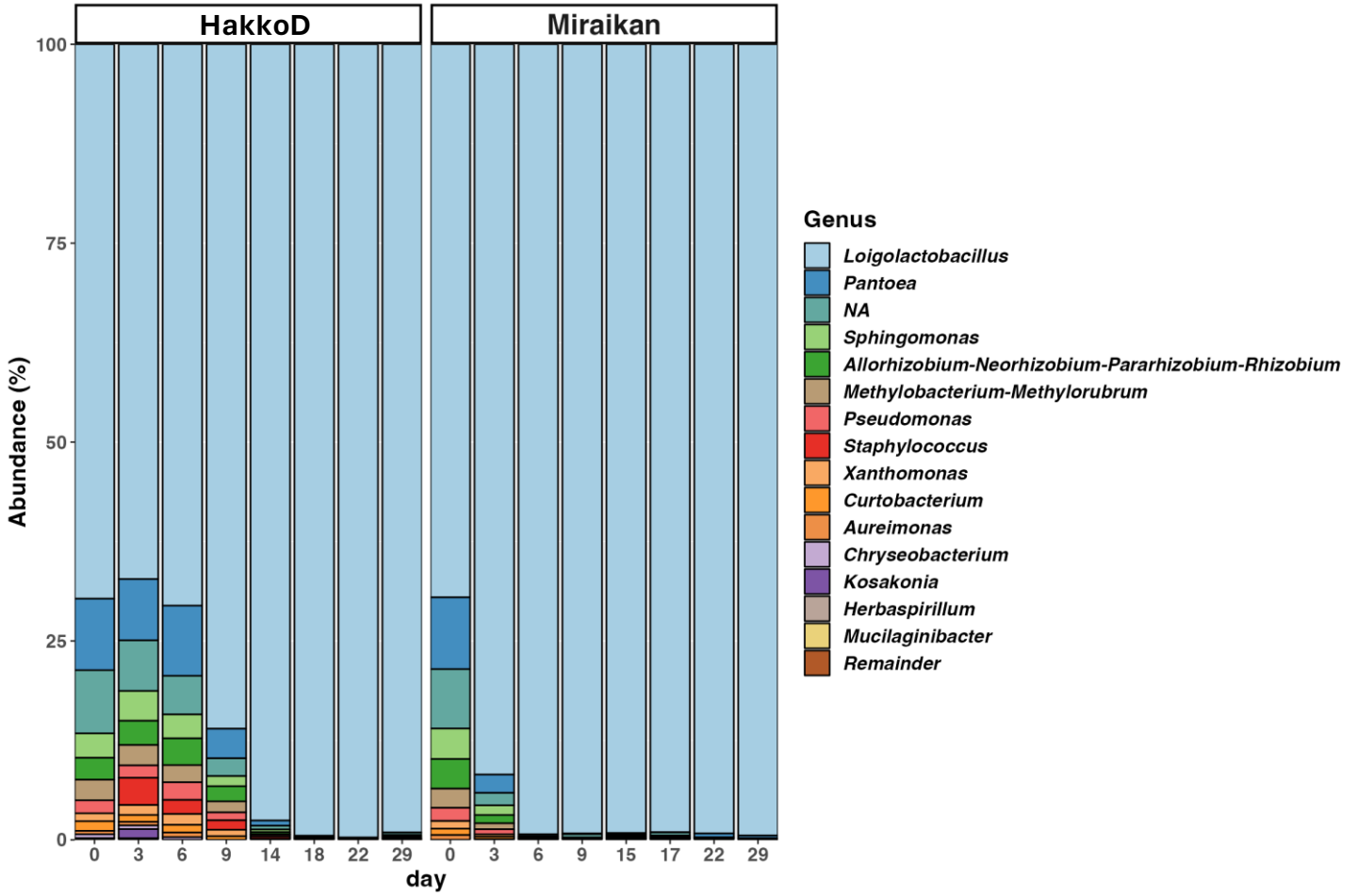
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Pantoea
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Sphingomonas
Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium
Methylobacterium-Methylorubrum
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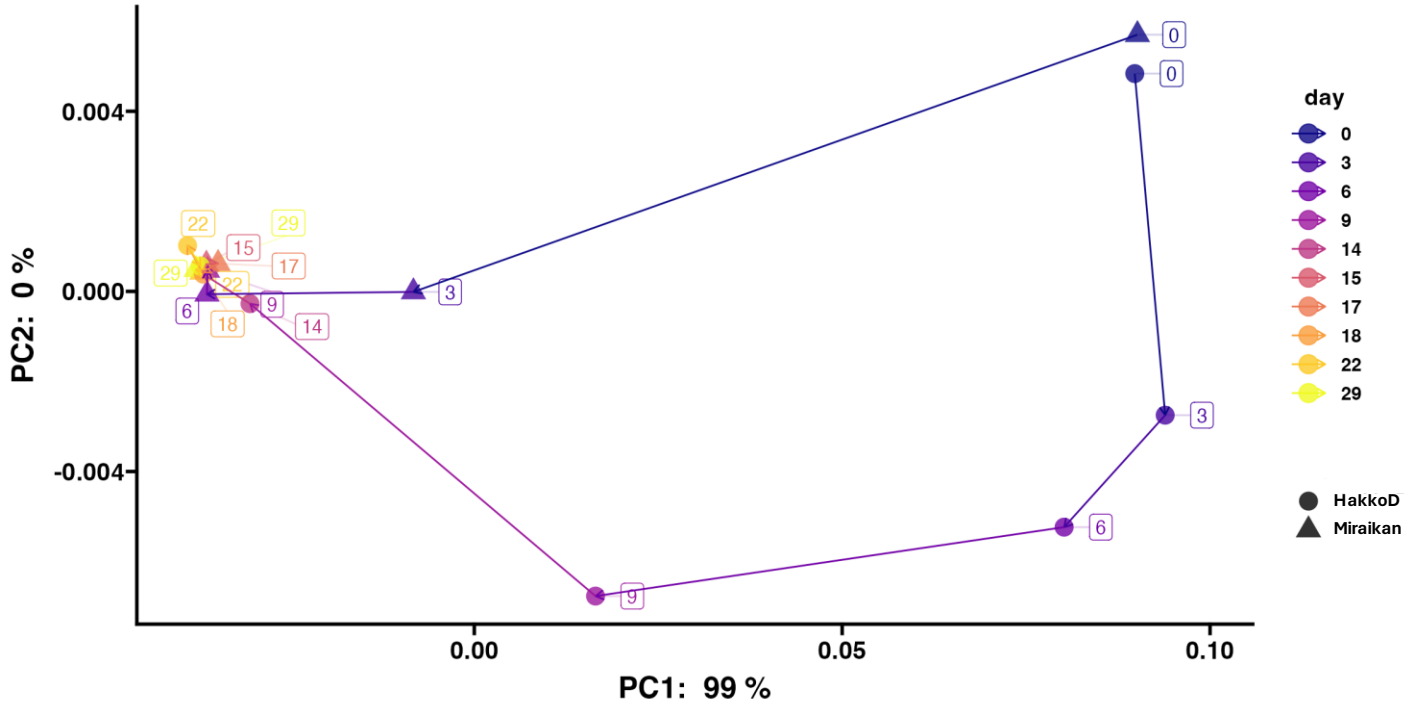
Remainder

(A)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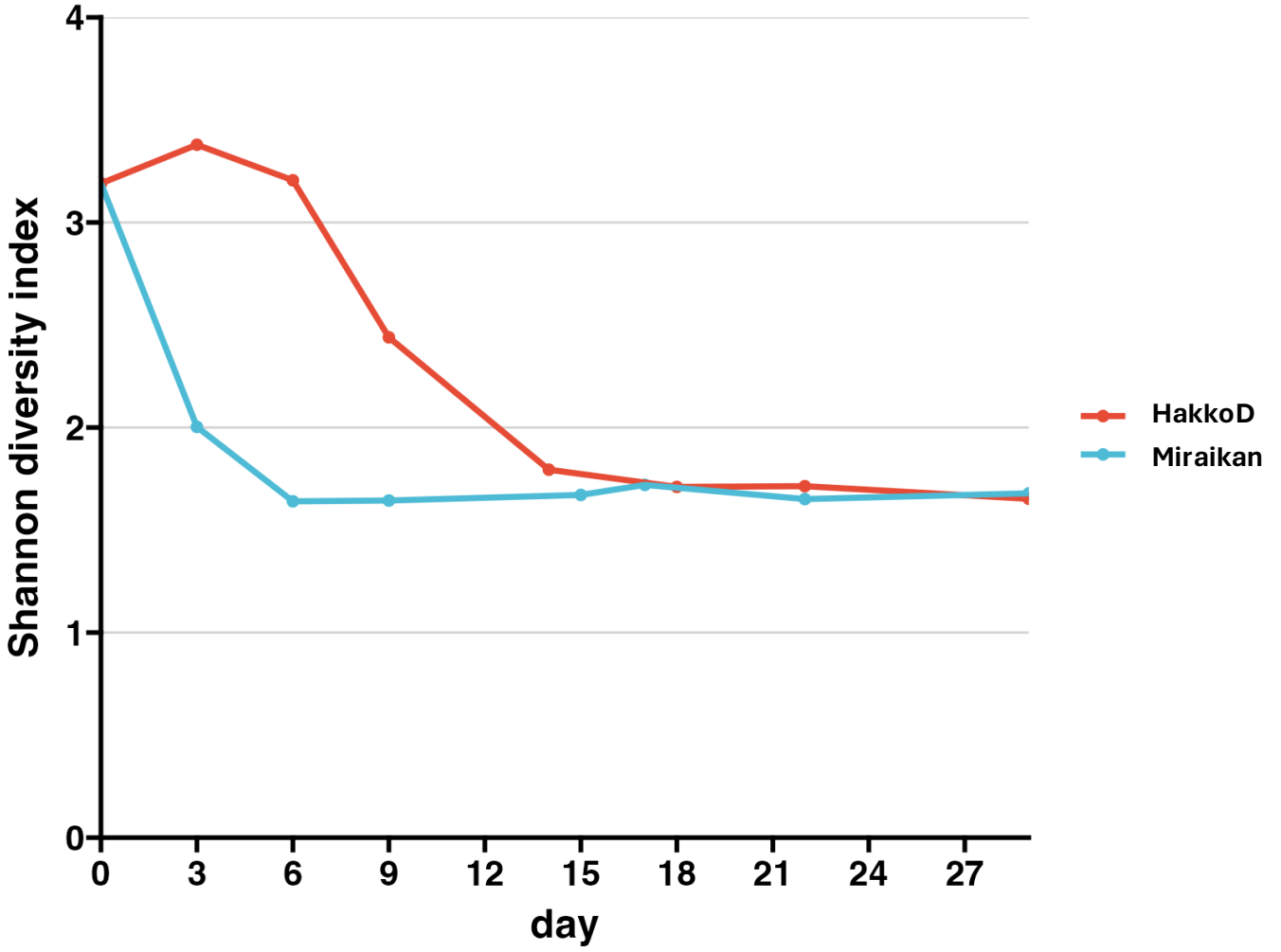
(B)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