Presence of an ultra-small microbiome in fermented cabbages (#82387)

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Presence of an ultra-small microbiome in fermented cabbages

Ji-Hyung Ha [Corresp., 1], **Hae-Won Lee** [1, 2], **So-Ra Yoon** [1], **Yun-Mi Dang** [1], **Miran Kang** [3], **Kwangho Lee** [4], **Jin-Woo Bae** [Corresp. 2]

[1] Hygienic Safety•Materials Research Group, World Institute of Kimchi, Gwangju, Republic of Korea

[2] Department of Biology and Department of Life and Nanopharmaceutical Sciences, Kyung Hee University, Seoul, Republic of Korea

[3] Practical Technology Research Group, World Institute of Kimchi, Gwangju, Republic of Korea

[4] Center for Research Facilities, Chonnam National University, Gwangju, Republic of Korea

Corresponding Authors: Ji-Hyung Ha, Jin-Woo Bae
Email address: hajee@wikim.re.kr, baejw@khu.ac.kr

Background. Ultramicrobacteria (UMB), also known as ultra-small bacteria, are tiny bacteria with a size less than 0.1 μm3. They have a high surface-to-volume ratio and are found in various ecosystems, including the human body. UMB can be classified into two types: one formed through cell contraction and the other that maintains a small size. The ultra-small microbiome (USM), which may contain UMB, includes all bacteria less than 0.2 μm in size and is difficult to detect with current methods. However, it poses a potential threat to food hygiene, as it can pass through sterilization filters and exist in a viable but non-culturable (VBNC) state. The data on the USM of foods is limited. Some bacteria, including pathogenic species, are capable of forming UMB under harsh conditions, making it difficult to detect them through conventional culture techniques.

Methods. The study described above focused on exploring the diversity of USM in fermented cabbage samples from three different countries (South Korea, China, and Germany). The samples of fermented cabbage (kimchi, suancai, and sauerkraut) were purchased and stored in chilled conditions until filtration. The filtration process involved two steps of Tangential Flow Filtration (TFF) using TFF cartridges with different pore sizes to separate normal size bacteria (NM) and USM. The USM and NM isolated via TFF were stored in a refrigerator at 4 °C until DNA extraction. The extracted DNA was then amplified using PCR and the full-length 16S rRNA gene was sequenced using single-molecule-real-time (SMRT) sequencing. TEM was used to confirm the presence of microorganisms in the USM of fermented cabbage samples

Results. To the best of our knowledge, this is the first study to identify the differences between USM and NM in fermented cabbages. Although the size of the USM was smaller than that of the NM, their diversity was not lower. In addition, some members in USM probably underwent cell shrinkage due to unfavorable environments, while others maintained their size. Major pathogens were not detected in the USM in fermented cabbages. Nevertheless, several potentially suspicious strains were detected. Our method can be used to screen food materials for the presence of USM undetectable via conventional methods. USM and NM were efficiently separated using tangential flow filtration and analyzed via single-molecule real-time sequencing. The USM in fermented vegetables differed from the conventional microbiome. This study could provide new insights into the ultra-small ecosystem in fermented foods, including fermented cabbages.

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[1] Hygienic Safety·Materials Research Group, World Institute of Kimchi, Gwangju, Republic of Korea
[2] Department of Biology and Department of Life and Nanopharmaceutical Sciences, Kyung Hee University, Seoul, Republic of Korea
[3] Practical Technology Research Group, World Institute of Kimchi, Gwangju, Republic of Korea
[4] Center for Research Facilities, Chonnam National University, Gwangju, Republic of Korea

Corresponding Author:
Ji-Hyoung Ha[1]
Department of Biology and Department of Life and Nanopharmaceutical Sciences, Kyung Hee University
Seoul 02447, Republic of Korea Email address: hajee@wikim.re.kr

Jin-Woo Bae[2]
Department of Biology and Department of Life and Nanopharmaceutical Sciences, Kyung Hee University
Seoul 02447, Republic of Korea Email address: baejw@khu.ac.kr

Abstract

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Introduction

Ultramicrobacteria (UMB) are less than 0.1 μm^3 (or less than 0.3 μm in diameter); the name UMB was first used by Torella and Morita (1981) to describe extremely small bacteria. UMB are also referred to as ultra-small bacteria, nanobacteria, nano-organisms, dwarf cells, ultramicro cells, nano-sized microorganisms, filterable bacteria, small low nucleic acid-content bacteria, and nanobes [(Velimirov, 2001; Duda et al., 2012; Ghuneim et al., 2018; Proctor et al., 2018). The small cell size of UMB provides a larger surface-to-volume ratio, thereby enabling the efficient absorption of nutrients in an oligotrophic environment (Giovannoni et al., 2005; Duda et al., 2012) and protecting against grazing pressure (Miteva & Brenchley, 2005; Williams et al., 2009). UMB exist in various ecosystems (Ghuneim et al., 2018), including the human body (Kajander & Ciftcioglu, 1998; He et al., 2015). While there is no official classification of UMB, they are classified into two types based on the effect of environmental factors on their morphology. The first type is UMB formed via cell contraction due to intrinsic and extrinsic factors, such as lack of nutrition or extremely harsh environments (Velimirov, 2001; Panikov, 2005), while the second type maintains a small size regardless of these factors and include strains different from existing taxa (Duda et al., 2012; Ghuneim et al., 2018). In this study, an ecosystem of bacteria less than 0.2 μm in size was confirmed; hence, filtrable bacteria including UMB and spores could be included. Therefore, we defined the ecosystem of all bacteria less than 0.2 μm in size as the ultra-small microbiome (USM). If a lethal pathogen is transformed into a UMB state, it is generally considered in a viable but non-culturable (VBNC) state (Kaprelyants et al., 1993). USM cannot be detected using the currently available culture-dependent methods and can

therefore pass through sterilization filters with a pore size of ≤ 0.2 μm (Nakai, 2020). Therefore, USM remains a potential threat to food hygiene.

Cabbages have various health benefits (Witzel et al., 2021). In particular, fermented cabbage can treat scurvy (Delf, 1918; Raak et al., 2014). A recent study has suggested that sulforaphane and lactic acid bacteria from fermented cabbage may help to lower the mortality rate of COVID-19 infections (Bousquet et al., 2021). Fermented cabbage is a popular item in Europe and North America, where it is processed and consumed as sauerkraut. In Asia, the type of cabbage preferred differs from that in Europe and is processed and consumed under the name "kimchi" in Korea and "suancai" in China. In El Salvador, cabbage is processed and consumed as "curtido."

To confirm the presence of USM in cabbage, we investigated various fermented cabbage types consumed in different regions and compared their microbial and UMB communities. To the best of our knowledge, this study is the first to examine USM inside fermented foods, such as fermented cabbage. This study employed a novel strategy by combining tangential-flow-filtration (TFF) and single-molecule-real-time (SMRT) sequencing to differentially detect and sequence the *16S rRNA* genes extracted from the ultra-small microbial communities. We aimed to distinguish between normal and ultra-small bacteria in fermented vegetables, and to identify the bacterial species with high resolution. Moreover, we attempted to understand the UMB community in food and identify potential pathogens, such as VBNC or persister states, that maximize the survival rate.

Materials & Methods

Fermented vegetable samples

To explore the diversity of USM in fermented cabbage, kimchi, a fermented cabbage made in South Korea; suancai, a fermented cabbage made in China; and sauerkraut, a fermented cabbage made in Germany, were purchased from an online market in 2019. Both kimchi and suancai samples were non-sterile, while sauerkraut was sterile. Notably, suancai contained a preservative (potassium sorbate). Kimchi was made from kimchi cabbage (*Brassica rapa* subsp. *pekinensis*), suancai was made from Chinese cabbage (*B. rapa* subsp. *pekinensis*), and sauerkraut was made from white cabbage (*Brassica oleracea* var. *capitata*). *B. rapa* subsp. *pekinensis*, produced in Korea and bred for kimchi production, is called kimchi cabbage in order to be easily distinguished from *B. rapa* subsp. *pekinensis*, which is used to produce suancai (CAC, 2013). The detailed ingredients of each fermented cabbage are shown in S1 Fig. The fermented cabbage samples were stored in chilled conditions at approximately 4 °C until filtration.

Pre-filtration

Pre-filtration was performed on the broths of samples to facilitate TFF using a polypropylene capsule filter (GVS Filter Technology, Morecambe, UK) with a pore size of 10 μm. A vacuum pump (2546C-10, Welch Materials Inc., Shanghai, China) was used to aid the filtration process by reducing the pressure during the pre-filtration step. Before pre-filtration, the polypropylene capsule filter and tubing were sterilized with a solution of sodium hypochlorite 0.1% (v/v).

TFF

TFF was performed using a TFF system (Cogent μScale TFF System; Millipore, Sigma-Aldrich, St. Louis, IL, USA) in two phases. In the first phase, a TFF cartridge (Pellicon 2 Mini Cassette, Media: Durapore 0.22 μm; Millipore, Sigma-Aldrich) was used to cut off particles > 0.2 μm for the isolation of normal size bacteria as well as to remove macromolecules, including plasmid DNA. In the second phase, a TFF cartridge (Pellicon 2 Mini Cassette, Media: Biomax 100 kDa; Millipore, Sigma-Aldrich) with a molecular weight cut-off (MWCO) of 100 kDa was used to remove small molecules below the USM size. Six samples, including (1) normal microbiome (NM) > 0.2 μm from kimchi (Kimchi_NM), (2) USM below 0.2 μm from kimchi (Kimchi_USM), (3) NM > 0.2 μm from sauerkraut (Sauerkraut_NM), (4) USM < 0.2 μm from sauerkraut (Sauerkraut_USM), (5) NM > 0.2 μm from suancai (Suancai_NM), and (6) USM < 0.2 μm from suancai (Suancai_USM), were subjected to further evaluation.

The samples were concentrated to approximately 25–50 fold via TFF followed by TFF system sterilization by recirculation with 0.1% (v/v) sodium hypochlorite for 30 min and cleaning by recirculation with sterilized ultrapure water for 2 h. The detailed specifications of the two phases of TFF are shown in S2 Fig. Additionally, Cai et al. used TFF to efficiently separate bacteria and viruses from the marine environment and found that the adsorption of bacterial cells in a filter made of polyvinylidene fluoride (PVDF) was low (Cai et al., 2015). Based on their results, we used a cassette filter based on PVDF (Durapore 0.22 μm) for TFF to separate the USM < 0.2 μm and the NM > 0.2 μm. The USM and NM isolated via TFF were stored in a refrigerator at 4 °C until DNA extraction.

DNA extraction and PCR amplification

Nucleic acids were extracted from samples using a DNeasy PowerSoil kit (Qiagen, Hilden, Germany) and quantified using the Quant-IT PicoGreen assay kit (Invitrogen, Thermo-Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Libraries were prepared via PCR amplification using the PacBio RS II. The nucleic acids were amplified with a primer set (27F, 5′- AGRGTTYGATYMTGGCTCAG-3′; 1492R, 5′-GGTTACCTTGTTACGACTT-3′) for the full-length 16S rRNA gene. The PCR conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, extension at 72 °C for 90 s, and a final extension at 72 °C for 5 min. Purification of the PCR amplicons was carried out using AMPure beads (Agencourt Bioscience, Beverly, MA, USA). To verify the amount and size of PCR products, fluorescence was measured using the Quant-IT PicoGreen assay kit (Thermo-Fisher Scientific), and the template size distribution was measured using an Agilent DNA 12000 kit (Agilent Technologies, Santa Clara, CA, USA). Pooled amplicons were used for library preparation PacBio Sequel sequencing. A library was prepared using the PacBio DNA template prep kit 1.0 (Pacific Biosciences, USA). The PacBio DNA sequencing kit 4.0 and 8 SMRT cells (Pacific Biosciences) were used for sequencing.

SMRT sequencing

SMRT sequencing was performed using a PacBio RSII system (Pacific Biosciences) according to the manufacturer's instructions. Circular consensus sequencing (CCS) reads, such as raw sequence reads, were processed using the SMRT analysis software (version 2.3, Pacific Biosciences). Short CCS reads and those with zero quality bases, considered as sequencing errors, were removed.

Taxonomic and statistical analysis

Taxonomic analysis of the CCS reads was performed using the MG-RAST server (Meyer et al., 2008) with the SILVA SSU database (Quast et al., 2013). The e-value, percent identity, minimal alignment length, and minimal abundance values were set to 5, 90, 15, and 1, respectively. Statistical analyses were performed using MicrobiomeAnalyst (Dhariwal et al., 2017). Data normalization for each sample was performed for total sum scaling. Abundance profiling was presented as a stacked bar chart by calculating the percentage abundance, and less than 10 taxa were omitted. Species richness based on the alpha diversity of samples was determined via rarefaction curve analysis (McMurdie & Holmes, 2013), and the diversity of operational taxonomic units (OTUs) was indicated by the Shannon index. A heat tree was constructed using the non-parametric Wilcoxon rank sum test and was used to statistically quantify the hierarchical structure of taxonomic classification (Foster et al., 2017). The distance method and principal coordinate analysis (PCoA) was used for determining the beta diversity of samples and were set to unweighted UniFrac distance and permutational multivariate analysis of variance (PERMANOVA), respectively.

In addition, the distance measure and clustering algorithm of the hierarchical clustering algorithm (HCA) were set to the Bray–Cutis index and Ward, respectively. A heat tree was used to compare and sum the microbial communities and UMB ultramicrobial communities for each sample at the species level. As a high-dimensional data analysis performed using a supervised machine learning algorithm, random forest classification analysis was conducted to identify the variability of strains in samples (Liaw & Wiener, 2002).

Transmission electron microscopy (TEM)

For TEM observations via negative staining, droplets of the samples were mounted on a carbon support film on a 150-mesh nickel grid, stained with 4% uranyl acetate for 10 min and 0.4% lead citrate for 6 min, washed three times with deionized water, and air-dried. In addition, the samples were prepared in the form of ultrathin sections. To this end, samples were fixed by the addition of glutaraldehyde and paraformaldehyde adjusted to 2% in 0.05 M phosphate buffer (pH 7.2) and then incubated at room temperature (15–25 °C) for 4.5 h under vacuum. The fixed samples were washed three times for 15 min each with 0.05 M phosphate buffer at pH 7.2. The washed samples were post-fixed with osmium tetroxide adjusted to 1% in 0.05 M phosphate buffer (pH 7.2) at room temperature for 1 h. The post-fixed samples were washed three times for 15 min each with 0.05 M phosphate buffer at pH 7.2 and then dehydrated by passing through an ethanol gradient from 50 to 100%. After dehydration, the samples were precipitated to resin (LR white resin; EMS, Hatfield, PA, USA), placed in a disposable mold and embedded for 24 h at 60 °C. After the sample was hardened, ultrathin sections were prepared using an ultramicrotome

equipped with a diamond knife and stained with 4% uranyl acetate for 10 min and 0.4% lead citrate for 6 min to complete sample preparation for TEM observation. The prepared samples were observed using a field-emission TEM (FE-TEM) (JEM-2100F; JEOL Ltd., Tokyo, Japan) at 200 kV accelerating voltage.

Data availability

The sequencing reads of fermented cabbages were deposited to the NCBI under BioProject ID PRJNA684410.

Results

SMRT sequencing

The NM and USM in fermented cabbage were separated using tangential flow filtration (TFF) and analyzed via SMRT sequencing. Sequence read information for each sample is presented in Table 1. A total of 19,356 sequence reads from Kimchi_NM, 1,383 from Kimchi_USM, 1,208 from Sauerkraut_NM, 1,610 from Sauerkraut_USM, 11,446 from Suancai_NM, and 1,570 from Suancai_USM were generated. The total number of reads from NM was much greater than that of USM. Notably, the number of reads from the Sauerkraut_USM was slightly higher than that from the Sauerkraut_NM. Kimchi_NM had the greatest number of sequence bases at 28,690,199 bp, while Sauerkraut_NM had the lowest at 1,741,058 bp. The average length of the reads was almost the same between groups, approximately 1,400 bp.

Taxonomic and statistical analyses

Community richness in the samples was expressed using the rarefaction curve (Fig 1A), while alpha diversity was expressed using the Shannon index (Fig 1B). Each sample in the rarefaction curve plateaued. Suancai_NM had the highest richness (OTUs) of 84), while Sauerkraut_NM had the lowest (OTUs of 11). However, generally, the OTUs in Kimchi_NM and Suancai_NM were more abundant than those in the Kimchi_USM, Sauerkraut_USM, and Suancai_USM, but not than that in Sauerkraut_NM. Since the rarefaction curve of Kimchi_NM was gently inclined, richness was high, while diversity was low. Based on the Shannon index, Suancai_NM had the highest microbial diversity, whereas Kimchi_NM had the lowest. The alpha diversity of suancai was higher than that of kimchi and sauerkraut when diversity was assessed between fermented cabbage types. Further, USM had higher alpha diversity on an average when diversity was assessed based on community type and regardless of the sample type.

The NM and USM in kimchi and suancai are shown in Fig 2A and S3 Fig. At the phylum level, Firmicutes were dominant in Kimchi_NM and Suancai_NM. In the former, Firmicutes accounted for 100% of the microbial community. Actinobacteria dominated Kimchi_USM, and uncultured bacteria dominated Sauerkraut_USM as well as Suancai_USM. At the species level, *Weissella koreensis* was dominant at 94% in Kimchi_NM, while *Weissella cibaria* was also present, but in minor quantities. *Cellulomonas uda* and *Cupriavidus pauculus* were predominant in Sauerkraut_NM, at 32% and 26%, respectively. However, in Sauerkraut_NM, uncultured bacteria accounted for a significant proportion (32%). *Lactobacillus acetotolerans* was dominant (53%) in Suancai_NM. *Lactobacillus similis* was also predominant in Suancai_NM, accounting

for 11%. *Cellulomonas biazotea* dominated at 42% in Kimchi_USM. In particular, candidate division TM7 single-cell isolate TM7a (TM7a), known as *Saccharibacteria*, was predominant at 35%. *Cellulomonas uda* predominated in Sauerkraut_USM at 36%. However, uncultured soil bacteria dominated Sauerkraut_USM at 54% and Suancai_USM at 48%.

The beta diversity of NM and USM in fermented cabbage samples was compared via PCoA (Fig 2B) and HCA (Fig 2C). The plot and dendrogram showed that Suancai_NM and Kimchi_USM were closely related as also observed for Sauerkraut_NM, Sauerkraut_USM, and Suancai_USM. In addition, PCoA indicated that Kimchi_NM was separated from the other samples. However, Suancai_NM, Kimchi_USM, and Kimchi_NM were grouped in the HCA dendrogram, as were Suancai_USM, Sauerkraut_NM, and Sauerkraut_USM.

A heat tree was used to compare and sum the NM and USM at the genus level for each fermented cabbage (Fig 3). The sum of NM and USM per fermented cabbage type is shown in Fig 3A–C. Obtaining this sum was equivalent to combining the NM and USM of each fermented cabbage in the bar chart of Fig 2A. Fig 3D–F compare the NM and USM in each fermented cabbage. In kimchi, several genera belonging to the phylum Firmicutes had a relatively high ratio in the NM compared to the USM (Fig 3D). Unlike in kimchi, in sauerkraut, several genera belonging to the phylum Proteobacteria had a relatively higher ratio in the NM compared to that in the USM (Fig 3E). Suancai was similar to kimchi as several genera belonging to the phylum Firmicutes had a relatively higher ratio in the NM compared to that in the USM (Fig 3F).

The random forest algorithm was used to confirm the top 15 OTUs with large variability between the microbiomes (Fig 4). The mean reduced accuracy represents the accuracy that the microbiome loses by excluding each variable. Thus, the lower the accuracy, more important the variable species is for a successful classification. Species are displayed in descending order of importance, that is, the higher the mean reduction accuracy, higher the importance of the species in the microbiome (Martinez-Taboada and Redondo, 2020). Since the mean decrease accuracy of uncultured bacteria was the highest (0.0279), the microbiome was likely to be divided based on the content of these strains. The mean decrease in accuracy of uncultured *Azospira* sp. and *Lactobacillus paracollinoides* was 0.0231 and 0.0230, respectively, which were the second and third highest, respectively.

Morphological observations via TEM

TEM was used to confirm the presence of microorganisms in the USM of fermented cabbage samples (Fig 5 and S4 Fig). Most of the USM were cocci with both outer and inner membranes observed in the USM isolated from all fermented cabbages. In addition, the periplasmic space between the outer and inner membranes was observed (Fig 5C, G, and K). The size of the microorganism in USM was approximately 100–200 nm. In addition, USM isolated from fermented cabbages had multiplied via dichotomy (Fig 5B and S4H, K Fig).

Discussion

In the present study, we identified the NM and USM in different fermented cabbages via TFF and SMRT to characterize and compare USM between cabbage types. TFF has been used to

concentrate various microorganisms in water and is an excellent technique for their separation or removal (Cai et al., 2015). TFF has shown 11–98% recovery for plankton viruses, smaller than 0.2 μm in size, from freshwater samples (Colombet et al., 2007). Therefore, in the present study, we employed TFF instead of conventional normal flow filtration (NFF) to separate and concentrate NM and USM from kimchi, sauerkraut, and suancai. TFF is more efficient than NFF since it can filter more liquid phase by smoothly removing the filter cake. In addition, while there were fewer sequence reads for USM than those for NM (Table 1), they might not have been obtained if not enriched via TFF.

The OTU values indicated that the species richness of NM is generally higher than that of USM (Fig 1). In particular, Suancai_NM exhibited the highest abundance and alpha diversity, as indicated by significantly higher OTU and Shannon index values of the microbiome compared to that of the other samples. For Kimchi_NM, the OTU value was relatively higher than that for other samples, yet its Shannon index value was the lowest. In addition, the rarefaction curve of Kimchi_NM increased only slightly, suggesting low diversity. The Shannon index was lower than the OTU value since *Weissella koreensis* showed > 94% dominance. In contrast, *L. acetotolerans* showed > 53% dominance in Suancai_NM, while other species showed minor dominance, between 1 and 11%. Therefore, the Shannon index was estimated to be the highest. The OTU values of USM were relatively low, with the one for Kimchi_USM being the lowest. However, its Shannon index was the second highest, > 1.6, possibly due to the even distribution of species. While the difference in alpha diversity values between the NM and USM of kimchi and suancai was high, the difference in alpha diversity value between the NM and USM of sauerkraut was small. Since sterilization was performed during the manufacturing process of sauerkraut, most of the normal microorganisms did not survive. Therefore, the difference in alpha diversity between Sauerkraut_NM and Sauerkraut_USM was not expected to be high. The species richness of USM was low, yet the diversity was higher than that of NM. The microbial distribution of the fraction that passed through the 0.2-μm filter, which is sterile, was more diverse than expected (Nakai, 2020).

Weissella koreensis, a dominant bacterium in kimchi (Jung et al., 2014), was first reported in kimchi in 2002 (Lee et al., 2002). Further, *Weissella* spp., including *W. koreensis*, are involved in kimchi fermentation (Cho et al., 2006). In line with previous reports, this study also showed that *W. koreensis* was highly prevalent in Kimchi_NM. However, in Kimchi_USM, *C. biazotea*, which is known to degrade cellulose (Rajoka & Malik, 1997), and TM7a, which is known to be parasitic on bacterial hosts (Marcy et al., 2007; Bor et al., 2019), were both detected.

The microbial abundance of USM and NM in each sample was relatively normalized via total sum scaling, yet since the number of sequence reads from Kimchi_USM was over ten times less than that of Kimchi_NM, *C. biazotea* and TM7a do not represent the microbiome in general kimchi containing USM and NM. In addition, based on the sequence reads, the precent ratio of USM to NM in kimchi was 7.1, which was the basis for the lower microbial abundance in USM compared with NM. Further, if the USM was not separated via TFF, *C. biazotea* and TM7a

would not have been identified in kimchi. We reviewed the available literature on the microbiome of kimchi and found no reports on *C. biazotea* and TM7a in kimchi via culture-dependent or culture-independent methods (Cho et al., 2006; Park et al., 2012; Jung et al., 2014; Lee et al., 2017; Maoloni et al., 2020; Park et al., 2020). The *16S rRNA* gene is shared among all bacteria and utilizing this gene would significantly reduce the labor and cost of profiling the identity and abundance of microorganisms in various environments, regardless of culture capacity (Lane et al., 1985; Woese, 1987; Olsen & Woese, 1993). However, the *16S rRNA* gene is not an optimal target, owing to the short read length of most commonly used sequencing platforms, such as Illumina, which limits the taxonomic resolution to families or genera (Earl et al., 2018; Jeong et al., 2021). However, if the entire *16S rRNA* gene is read via SMRT sequencing, which can also analyze long sequences, taxonomic resolution can be improved. In a previous study, 60% of specific phyla, such as the phylum Microgenomates, were not detected via PCR using the 518F and 806R primer sets (Brown et al., 2016), and the low taxonomic resolution due to short sequencing limits the amount of data on microbial ecology. Therefore, the identification of *C. biazotea* and TM7a in kimchi may be attributed to the use of SMRT sequencing in the current study. In particular, TM7, also known as *Saccharibacteria*, have been reported in the oral cavity (Bor et al., 2019) and are known to be ultra-small (200–300 nm) and parasitic bacteria attached to the surface of host bacteria (Bor et al., 2016), which complicates their detection via conventional culture methods. Further, TM7 do not grow unless a special method of symbiosis is employed (Murugkar et al., 2020). The causal relationship observed herein remains unclear, and further studies are warranted to determine how TM7 is transmitted to humans. In addition to *C. biazotea* and TM7a, other bacteria were rarely found in Kimchi_USM. Therefore, most of the bacteria in the USM did not adapt to the kimchi environment or lost their survival competition to *Weissella* spp.

Sauerkraut_NM and Sauerkraut_USM were dominated by *C. uda*. Like *C. biazotea, C. uda* secretes cellulases. The genus *Cellulomonas* is abundant in soil (Robinson, 2014) and was found in Sauerkraut_NM and Sauerkraut_USM, probably derived from the soil where the raw cabbage had been planted. In addition, it is assumed that *Cellulomonas* spp., distributed in very small numbers in Suancai_USM, are also derived from soil. Martin et al. discovered novel USM from the class Actinobacteria, belonging to the genus *Cellulomonas*, in five freshwater reservoirs (Hahn et al., 2003). Although only Actinobacteria living in freshwater environments were mentioned, *Cellulomonas* in soil should also have several USM types. *Cupriavidus pauculus* (also known as *Ralstonia paucula*), detected only in Sauerkraut_NM, can pass ultrafiltration (Cuadrado et al., 2010), although it was not found in Sauerkraut_USM. In addition, *C. pauculus* may not occur in a USM, because it is a filterable bacterium.

Suancai_NM was dominated by *L. acetotolerans*, which was first reported in fermented vinegar broth (Entani et al., 1986). *Lactobacillus* spp. are involved in suancai fermentation (Liu et al., 2019). However, *L. acetotolerans* was previously reported as abundant in pao cai, but not in suancai (Cao et al., 2017; Liu et al., 2019). This disparity is possibly attributed to the differences between the manufacturing areas (such as temperature, salinity, and seasoning).

Uncultured bacteria, including soil bacteria, were dominant in Suancai_USM, and these OTUs may not be included in the SILVA SSU database. Similarly, in Sauerkraut_NM and Sauerkraut_USM, uncultured soil bacteria and uncultured bacteria were also dominant, while random forest analysis indicated that their OTUs exhibited large variation among samples (Fig 4).

Ralstonia spp. were detected in both Sauerkraut_USM and Suancai_USM at < 1%. However, *Ralstonia* spp. are likely not UMB as they are filterable bacteria, as in the case of *C. pauculus*. In addition, since they can survive by attaching to the ultrapure water system (Kulakov et al., 2002), they may not be resident microorganisms in kimchi or suancai. Detected at < 1%, *Ralstonia* spp. do not present a major bias. Nevertheless, a more thorough sterilization may be necessary when similar studies are conducted in the future.

Comparison of the microbial and ultramicrobial communities of kimchi, sauerkraut, and suancai via PCoA and HCA (Fig 2B and 2C) showed differences between them. In addition, although each fermented cabbage had distinct microbial ecology (Fig 3A–C), the heat tree indicated differences between sample NM and USM (Fig 3D–F). While the main ingredients (*Brassica rapa* subsp. *pekinensis*) of kimchi and suancai are similar, differences in the manufacturing method, other ingredients, seasoning, and the surrounding environment might have contributed to the prevalence of different microbial and ultramicrobial communities. Since sauerkraut was sterilized in the manufacturing process, the difference between the microbial and ultramicrobial communities was not high, and was very similar to Suancai_USM. In sauerkraut, the genes of both NM and USM were mixed with those of dead cells due to sterilization; the reason behind the microbial abundance not being high, even though the genes of the dead cells were mixed, is presumed to be that the genome of the dead bacteria was decomposed during the sterilization process.

Although we successfully identified USM between 0.2 μm and 100 kDa in fermented vegetables, TFF and SMRT sequencing methodologies may lead to misrecognition of fragments of bacteria (Duda et al., 2011). Therefore, we sought to confirm the presence of ultra-small microorganism in USM via TEM (Fig 5 and S4 Fig). The ultra-small microorganism in Kimchi_USM, Sauerkraut_USM, and Suancai_USM were observed as coccoid types, 100–200 nm in size, with outer and inner membranes, as well as multiplication via dichotomy; therefore, the existence of ultra-small microorganism in the UMB of fermented cabbages was confirmed. The possibility that it is an outer membrane vesicle cannot be excluded (Cecil et al., 2019). However, bisection was observed; therefore, the possibility that it is an ultra-small microorganism cannot be ruled out.

Rod shape bacteria such as genera *Cellulomonas*, *Cupriavidus*, and *Lactobacillus* were also detected in USM using SMRT, while only spherical shape bacteria were found in TEM. Since, as previously suggested, spherical shaped bacteria must be at least 250–300 nm in diameter to maintain the 250–300 proteins essential for life, the normal state may therefore be rod shape; however, it might have transformed into a spherical shape for survival (Ghuneim et al., 2018). Moreover, USM formation, even in nutrient-rich environments, was possibly

attributed to the action of predators and selective pressures such as pH and drying (Pernthaler et al., 2001; Simon et al., 2002; Pernthaler, 2017).

Major human pathogens were not found among the NM or USM of the fermented cabbages. Although the OTU and sequence reads were small, several taxa suspected of causing human disease, such as *Ralstonia* (due to possible contamination during the experiment), were detected in USM, and a significant proportion of uncultured bacteria, with or without pathogenicity, were detected.

If non-sterilized vegetables are fermented in a place with poor hygiene, a potential smaller VBNC state may develop in unfavorable environments, such as low pH and high osmotic concentration of fermented vegetables. The VBNC state is a survival mechanism that protects bacteria from adverse environmental conditions (Ramamurthy et al., 2014). In addition, VBNC bacteria are considered dormant and can be detected in almost any ecological niche (Ayrapetyan & Oliver, 2016), however, their detection via conventional culture methods is challenging, as they do not grow, or exhibit significantly slow growth rates (Oliver, 2005; Ayrapetyan & Oliver, 2016). Nevertheless, the USM and NM differed between fermented cabbages, with some bacteria detected in their original UMB form, as in the case of TM7a. Ultra-small microorganism in the VBNC state must have also been present in an environment where growth seemed difficult due to the dominance of lactic acid bacteria.

VBNC pathogens in foods can withstand extreme stress conditions, including starvation, pasteurization processes, and antibiotics (Chaveerach et al., 2003; Li et al., 2014). The VBNC state of bacteria can be induced by extreme stress (Ayrapetyan & Oliver, 2016). Further, VBNC bacteria, known to be similar to persistent bacteria, can exist stochastically in a rapidly growing environment (Ayrapetyan et al., 2015; Goncalves & de Carvalho, 2016; Orman et al., 2016). Notably, some bacterial subpopulations can withstand rapid-onset stress, suggesting that they do not rely on time-dependent alterations in gene expression as a response to stressors (Ayrapetyan & Oliver, 2016). Overall, the VBNC phenomenon is of major significance for food hygiene (Dong et al., 2020). If the size of bacteria is reduced to ultra-small size owing to various unfavorable factors, they are generally assumed to enter the VBNC state. While ultra-small sized bacteria can be considered in the VBNC state, the opposite cannot be assumed as there are cases where size increases upon entering the VBNC state.

Conclusions

In the present study, we utilized SMRT sequencing and TFF to investigate the diversity of USM and NM in fermented cabbage products manufactured in Korea, Germany, and China. The fermented cabbage products analyzed herein are manufactured through distinct methods, in accordance with the regulations and culture of respective regions. In the case of kimchi, the main ingredients are salted, seasoned, and then fermented without sterilization. In contrast, the main and sub-ingredients of sauerkraut and suancai are salted together and sterilized or preservatives are added to reduce pathogenic microorganisms and increase the shelf-life of cabbage. These seemingly similar yet distinct manufacturing approaches resulted in differences between the

microbial communities in cabbages. Ultramicrobial and microbial communities were efficiently separated via TFF and analyzed through SMRT sequencing. We differentiated between NM and USM in fermented cabbage, identifying individual bacteria at species-level resolution. The USM in cabbage were different from NM. While major pathogens were not observed within USM, several suspicious strains were detected. Therefore, if pathogenic species are present within the manufacturing environment owing to poor sanitization, ultra-small microorganism formed due to the extreme fermentation environment are likely to be found in fermented food products, which poses a threat to consumer health. Taken together, the current study provides novel insights into the USM in foods, particularly fermented cabbage.

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Figure 1

Figure 1. Rarefaction curve (A) and Shannon index (B) of microbial and ultramicrobial communities detected in fermented cabbages.

Relationship between number of operational taxonomic units (OTUs) and sequences was applied to a rarefaction curve, and each sample was plateaued. Shannon index was expressed for each sample (left), cabbage type (middle), and community (right).

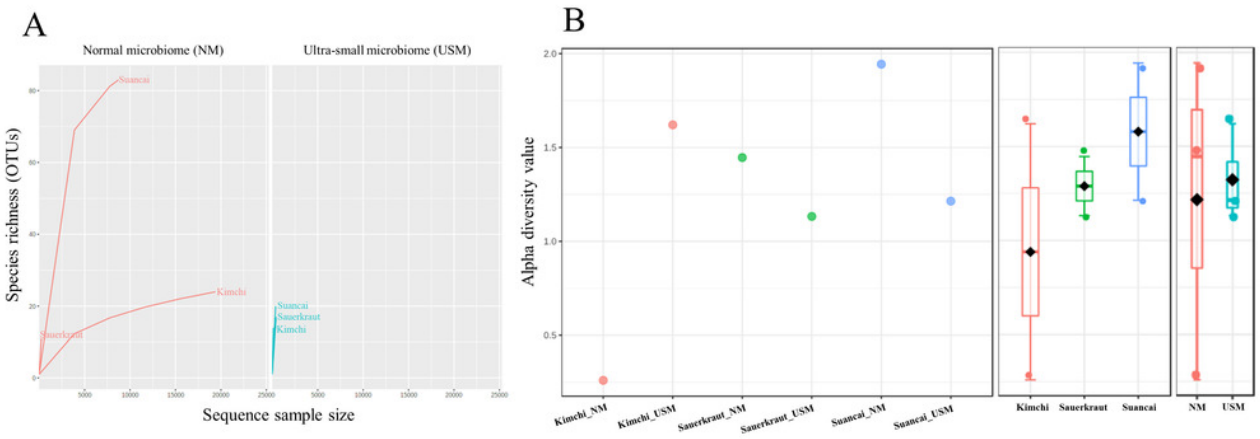

Figure 2

Figure 2. Relative abundance profiling (A), principal coordinate analysis (PCoA) plot (B), and hierarchical cluster analysis (HCA) dendrogram (C) reflected the species-level abundance

OTUs with an abundance below 10 as determined via relative abundance profiling were expressed as others. The statistical significance of the clustering pattern in the PCoA plot was evaluated through permutational ANOVA (PERMANOVA). The distance measure and clustering algorithm of HCA were applied to the Bray–Cutis index and Ward, respectively.

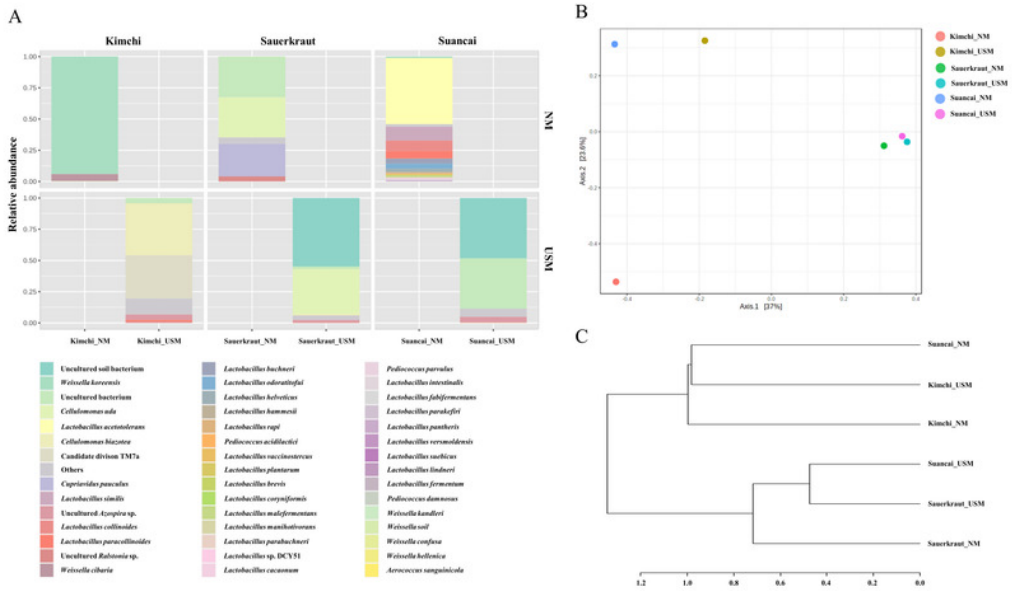

Figure 3

Figure 3. A heat tree used to compare and sum the microbial and ultramicrobial communities for each sample at the genus level.

A–C: the total microbiome of kimchi, sauerkraut, and suancai, respectively, D–F: comparison between the NM and USM of kimchi, sauerkraut, and suancai, respectively.

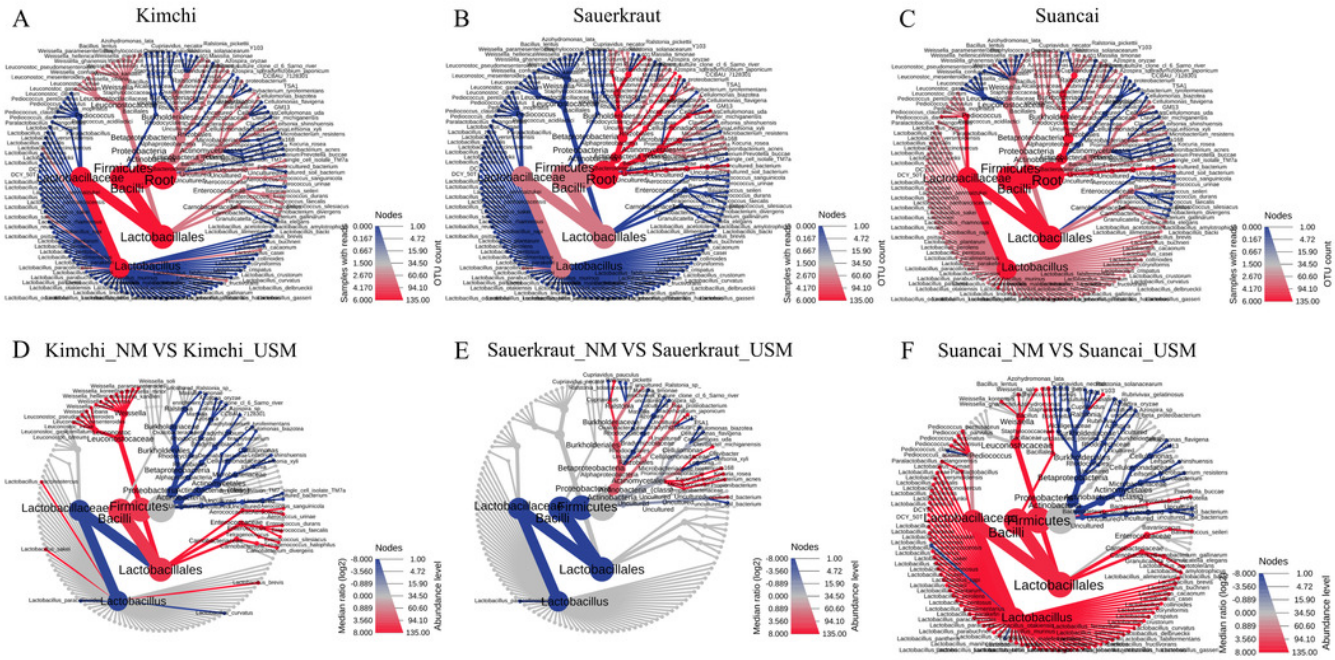

Figure 4

Figure 4. Random forest classification analysis.

Analysis confirmed the top 15 OTUs with greatest variability between communities. The mean reduced accuracy represents the accuracy that communities lose by excluding each variable. The lower the accuracy, more important the variable species for a successful classification.

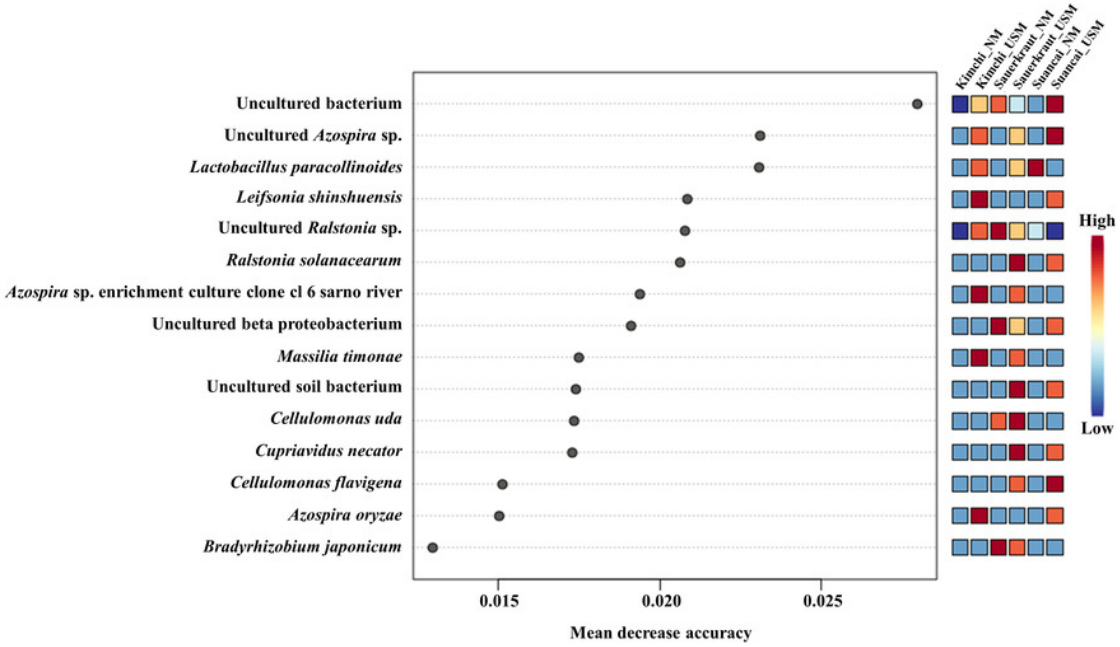

Figure 5

Figure 5. Transmission electron micrographs for ultramicrobial communities after ultra-section of fermented cabbages.

Transmission electron micrographs of the ultramicrobial community of a size < 0.2 μm in (A–D) kimchi (Kimchi_USM), (E–H) sauerkraut (Sauerkraut_USM), and (I–L) suancai (Suancai_USM). DT, dichotomy; IM, inner membrane; PS, periplasmic space: OM, outer membrane.

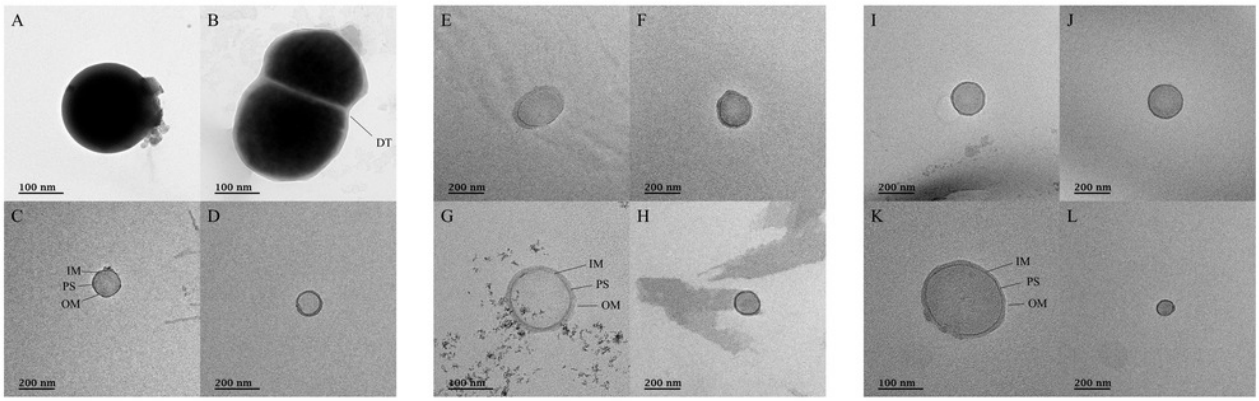

Table 1(on next page)

Table 1. States of sequence reads for each sample after trimming.

Kimchi_NM, normal microbiome > 0.2 μm in size from kimchi; Kimchi_USM, ultra-small microbiome < 0.2 μm in size from kimchi; Sauerfkraut_NM, normal microbiome > 0.2 μm in size from sauerkraut; Sauerkraut_USM, ultra-small microbiome < 0.2 μm in size from sauerkraut; Suancai_NM, normal microbiome > 0.2 μm in size from suancai; Suancai_USM, ultra-small microbiome < 0.2 μm in size from suancai.

Table 1. States of sequence reads for each sample after trimming.

Sample name	Read counts	Total read bases (bp)	Average read length (bp)
Kimchi_NM	19,356	28,690,199	1,482
Kimchi_USM	1,383	1,983,758	1,434
Sauerkraut_NM	1,208	1,741,058	1,441
Sauerkraut_USM	1,610	2,293,503	1,482
Suancai_NM	11,446	16,750,589	1,463
Suancai_USM	1,570	2,237,604	1,425

Kimchi_NM, normal microbiome > 0.2 μm in size from kimchi; Kimchi_USM, ultra-small microbiome < 0.2 μm in size from kimchi; Sauerfkraut_NM, normal microbiome > 0.2 μm in size from sauerkraut; Sauerkraut_USM, ultra-small microbiome < 0.2 μm in size from sauerkraut; Suancai_NM, normal microbiome > 0.2 μm in size from suancai; Suancai_USM, ultra-small microbiome < 0.2 μm in size from suancai.