Presence of an ultra-small microbiome in fermented cabbages

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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 at approximately 4 °C until filtration. The filtration process involved two steps of Tangential Flow Filtration (TFF) using TFF cartridges with different pore sizes (0.2 μm and 100 kDa) 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. The transmission electron microscope (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 (average 2,171,621 bp) was smaller than that of the NM (average 15,727,282 bp), diversity in USM (average H ' = 1.32) was not lower than that in NM (average H ' = 1.22). 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 (genera *Cellulomonas* and *Ralstonia*) 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 of fermented vegetables exhibited differences in size, diversity, and composition compared with the conventional microbiome. This study could provide new insights into the ultra-small ecosystem in fermented foods, including fermented cabbages.

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26 **Abstract**
27 **Background.** Ultramicrobacteria (UMB), also known as ultra-small bacteria, are tiny bacteria
28 with a size less than 0.1 μm^3. They have a high surface-to-volume ratio and are found in various
29 ecosystems, including the human body. UMB can be classified into two types: one formed
30 through cell contraction and the other that maintains a small size. The ultra-small microbiome
31 (USM), which may contain UMB, includes all bacteria less than 0.2 μm in size and is difficult to
32 detect with current methods. However, it poses a potential threat to food hygiene, as it can pass
33 through sterilization filters and exist in a viable but non-culturable (VBNC) state. The data on the
34 USM of foods is limited. Some bacteria, including pathogenic species, are capable of forming
35 UMB under harsh conditions, making it difficult to detect them through conventional culture
36 techniques.
37 **Methods.** The study described above focused on exploring the diversity of USM in fermented
38 cabbage samples from three different countries (South Korea, China, and Germany). The
39 samples of fermented cabbage (kimchi, suancai, and sauerkraut) were purchased and stored in

40   chilled conditions at approximately 4 °C until filtration. The filtration process involved two steps
41   of Tangential Flow Filtration (TFF) using TFF cartridges with different pore sizes (0.2 μm and
42   100 kDa) to separate normal size bacteria (NM) and USM. The USM and NM isolated via TFF
43   were stored in a refrigerator at 4 °C until DNA extraction. The extracted DNA was then
44   amplified using PCR and the full-length 16S rRNA gene was sequenced using single-molecule-
45   real-time (SMRT) sequencing. Transmission electron microscope (TEM) was used to confirm
46   the presence of microorganisms in the USM of fermented cabbage samples.
47   **Results.** To the best of our knowledge, this is the first study to identify the differences between
48   USM and NM in fermented cabbages. Although the size of the USM (average 2,171,621 bp) was
49   smaller than that of the NM (average 15,727,282 bp), diversity in USM (average H ' = 1.32) was
50   not lower than that in NM (average H ' = 1.22). In addition, some members in USM probably
51   underwent cell shrinkage due to unfavorable environments, while others maintained their size.
52   Major pathogens were not detected in the USM in fermented cabbages. Nevertheless, several
53   potentially suspicious strains (genera *Cellulomonas* and *Ralstonia*) were detected. Our method
54   can be used to screen food materials for the presence of USM undetectable via conventional
55   methods. USM and NM were efficiently separated using TFF and analyzed via SMRT
56   sequencing. The USM of fermented vegetables exhibited differences in size, diversity, and
57   composition compared with the conventional microbiome. This study could provide new insights
58   into the ultra-small ecosystem in fermented foods, including fermented cabbages.
59

Introduction

61   Ultramicrobacteria (UMB) are less than 0.1 μm^3 (or less than 0.3 μm) in diameter; the name
62   UMB was first used by Torella and Morita (1981) to describe extremely small bacteria. UMB are
63   also referred to as ultra-small bacteria, nanobacteria, nano-organisms, dwarf cells, ultramicro
64   cells, nano-sized microorganisms, filterable bacteria, small low nucleic acid-content bacteria, and
65   nanobes (Velimirov, 2001; Duda et al., 2012; Ghuneim et al., 2018; Proctor et al., 2018). The
66   small cell size of UMB provides a larger surface-to-volume ratio, thereby enabling the efficient
67   absorption of nutrients in an oligotrophic environment (Giovannoni et al., 2005; Duda et al.,
68   2012) and protecting against grazing pressure (Miteva & Brenchley, 2005; Williams et al.,
69   2009). Therefore, UMB exist in various ecosystems, such as subterranean bedrock, soil, ocean,
70   and river (Ghuneim et al., 2018), and the human body (Kajander & Ciftcioglu, 1998; He et al.,
71   2015). While there is no official classification of UMB, they are classified into two types based
72   on the effect of environmental factors on their morphology. The first type is UMB formed via
73   cell contraction due to intrinsic and extrinsic factors, such as lack of nutrition or extremely harsh
74   environments (Velimirov, 2001; Panikov, 2005), while the second type maintains a small size
75   regardless of these factors and include strains different from existing taxa (Duda et al., 2012;
76   Ghuneim et al., 2018). In this study, an ecosystem of bacteria less than 0.2 μm in size was
77   confirmed; hence, filtrable bacteria including UMB and spores could be included. Therefore, we
78   defined the ecosystem of all bacteria less than 0.2 μm in size as the ultra-small microbiome
79   (USM). If a lethal pathogen is transformed into a UMB state, it is generally considered in a

80   viable but non-culturable (VBNC) state (Kaprelyants et al., 1993). USM cannot be detected
81   using the currently available culture-dependent methods and can therefore pass through
82   sterilization filters with a pore size of ≤ 0.2 μm (Nakai, 2020). Therefore, USM remains a
83   potential threat to food hygiene (Lee et al., 2022a).
84   Cabbages have various health benefits (Witzel et al., 2021). In particular, fermented
85   cabbage can treat scurvy (Raak et al., 2014). A recent study has suggested that sulforaphane and
86   lactic acid bacteria from fermented cabbage may help to lower the mortality rate of COVID-19
87   infections (Bousquet et al., 2021). Fermented cabbage is a popular item in Europe and North
88   America, where it is processed and consumed as sauerkraut. In Asia, the type of cabbage
89   preferred differs from that in Europe and is processed and consumed under the name "kimchi" in
90   Korea and "suancai" in China. In El Salvador, cabbage is processed and consumed as "curtido"
91   (Lee et al., 2022a).
92   To confirm the presence of USM in cabbage, this study investigated various types of
93   fermented cabbage consumed in different regions and compared their microbial and
94   ultramicrobial communities. Because fermented cabbage can greatly vary depending on the
95   region, manufacturing method, and cultural tradition, studying only one type of fermented
96   cabbage would not be representative. Therefore, this study selected samples from kimchi,
97   sauerkraut, and suancai, which are representative of fermented cabbage in other regions, to
98   ensure a comprehensive analysis. In addition, this study employed a novel strategy by combining
99   tangential flow filtration (TFF) and single-molecule-real-time (SMRT) sequencing to
100   differentially detect and sequence the 16S rRNA genes extracted from the ultra-small microbial
101   communities.
102   We aimed to distinguish between normal size bacteria (NM) and ultra-small bacteria in
103   fermented vegetables and identify the bacterial species with high resolution. Moreover, we
104   attempted to understand the USM in food and identify potential pathogens, such as VBNC or
105   persister states, which maximize the survival rate. To the best of our knowledge, this study is the
106   first to examine the presence of USM in fermented foods, such as fermented cabbage, providing
107   new insights into the potential risks associated with their presence.
108
109   **Materials & Methods**
110   **Fermented vegetable samples**
111   To explore the diversity of USM in fermented cabbage, kimchi, a fermented cabbage made in
112   South Korea; suancai, a fermented cabbage made in China; and sauerkraut, a fermented cabbage
113   made in Germany, were purchased (8 kg of kimchi, 4 kg of sauerkraut, and 5 kg of suancai) from
114   an online market in June 2019. Moreover, the expiration date of kimchi, sauerkraut, and suancai
115   from the date of manufacture was 1, 36, and 18 months, respectively. Both kimchi and suancai
116   samples were non-sterile, while sauerkraut was sterile. Notably, suancai contained a preservative
117   (potassium sorbate). Kimchi was made from kimchi cabbage (*Brassica rapa* subsp. *pekinensis*),
118   suancai was made from Chinese cabbage (*B. rapa* subsp. *pekinensis*), and sauerkraut was made
119   from white cabbage (*Brassica oleracea* var. *capitata*). *B. rapa* subsp. *pekinensis*, produced in

120 Korea and bred for kimchi production, is called kimchi cabbage in order to be easily
121 distinguished from *B. rapa* subsp. *pekinensis*, which is used to produce suancai (CAC, 2013). In
122 addition, the fermented cabbages used in this study were not artificially produced in a laboratory,
123 but were representative commercial products commonly consumed by the general public. The
124 detailed ingredients of each fermented cabbage are shown in *Fig. S1*. The fermented cabbage
125 samples were stored in chilled conditions at approximately 4 °C until filtration (Lee et al.,
126 2022a).
127 **Pre-filtration**
128 Pre-filtration was performed on the broths of samples to facilitate TFF using a polypropylene
129 capsule filter (GVS Filter Technology, Morecambe, UK) with a pore size of 10 μm. A vacuum
130 pump (2546C-10, Welch Materials Inc., Shanghai, China) was used to aid the filtration process
131 by reducing the pressure during the pre-filtration step. Before pre-filtration, the polypropylene
132 capsule filter and tubing were sterilized with a solution of sodium hypochlorite 0.1% (v/v) (Lee
133 et al., 2022a).
134 **TFF**
135 TFF was performed using a TFF system (Cogent μScale TFF System; Millipore, Sigma-Aldrich,
136 St. Louis, IL, USA) in two phases. In the first phase, a TFF cartridge (Pellicon 2 Mini Cassette,
137 Media: Durapore 0.22 μm; Millipore, Sigma-Aldrich) was used to cut off particles > 0.2 μm for
138 the isolation of normal size bacteria as well as to remove macromolecules, including plasmid
139 DNA. In addition, because UMB can pass through a 0.2-μm pore size filter due to its small size
140 (Cavicchioli & Ostrowski, 2003; Duda et al., 2012; Nakai, 2020), a 0.2-μm pore size filter was
141 used in many previous studies to identify UMB present in various environments (Silbaq, 2009;
142 Loveland-Curtze et al., 2010; Sahin et al., 2010; Maejima et al, 2018). Therefore, we set the pore
143 size of the filter separating USM from NM to 0.2 μm to confirm USM based on UMB. Although
144 USM encompasses endospores and OMVs including UMB, separation standard for the
145 microbiome was set as UMB. In the second phase, a TFF cartridge (Pellicon 2 Mini Cassette,
146 Media: Biomax 100 kDa; Millipore, Sigma-Aldrich) with a molecular weight cut-off (MWCO)
147 of 100 kDa was used to remove small molecules below the USM size. Six samples, including (1)
148 normal microbiome (NM) > 0.2 μm from kimchi (Kimchi_NM), (2) USM below 0.2 μm from
149 kimchi (Kimchi_USM), (3) NM > 0.2 μm from sauerkraut (Sauerkraut_NM), (4) USM < 0.2 μm
150 from sauerkraut (Sauerkraut_USM), (5) NM > 0.2 μm from suancai (Suancai_NM), and (6)
151 USM < 0.2 μm from suancai (Suancai_USM), were subjected to further evaluation.
152   The samples were concentrated to approximately 25–50 fold via TFF followed by TFF
153 system sterilization by recirculation with 0.1% (v/v) sodium hypochlorite for 30 min and
154 cleaning by recirculation with sterilized ultrapure water for 2 h. The detailed specifications of the
155 two phases of TFF are shown in *Fig. S2*. Additionally, Cai and colleagues (Cai et al, 2015) used
156 TFF to efficiently separate bacteria and viruses from the marine environment and found that the
157 adsorption of bacterial cells in a filter made of polyvinylidene fluoride (PVDF) was low (Cai et
158 al., 2015). Based on their results, we used a cassette filter based on PVDF (Durapore 0.22 μm)

159   for TFF to separate the USM < 0.2 μm and the NM > 0.2 μm. The USM and NM isolated via

160   TFF were stored in a refrigerator at 4 °C until DNA extraction (Lee et al., 2022a).

161   **DNA extraction and PCR amplification**

162   Nucleic acids were extracted from 5 to 10 mL of concentrated NM and USM sample solutions

163   using a DNeasy PowerSoil kit (Qiagen, Hilden, Germany) and quantified using the Quant-IT

164   PicoGreen assay kit (Invitrogen, Thermo-Fisher Scientific, Waltham, MA, USA) following the

165   manufacturer's instructions. Libraries were prepared via PCR amplification using the PacBio RS

166   II. The nucleic acids were amplified with a primer set (27F, 5′-

167   AGRGTTYGATYMTGGCTCAG-3′; 1492R, 5′-GGTTACCTTGTTACGACTT-3′) for the full-

168   length 16S rRNA gene. The PCR conditions were as follows: initial denaturation at 94 °C for 5

169   min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s,

170   extension at 72 °C for 90 s, and a final extension at 72 °C for 5 min. Purification of the PCR

171   amplicons was carried out using AMPure beads (Agencourt Bioscience, Beverly, MA, USA). To

172   verify the amount and size of PCR products, fluorescence was measured using the Quant-IT

173   PicoGreen assay kit (Thermo-Fisher Scientific), and the template size distribution was measured

174   using an Agilent DNA 12000 kit (Agilent Technologies, Santa Clara, CA, USA). Pooled

175   amplicons were used for library preparation PacBio Sequel sequencing. A library was prepared

176   using the PacBio DNA template prep kit 1.0 (Pacific Biosciences, USA). The PacBio DNA

177   sequencing kit 4.0 and 8 SMRT cells (Pacific Biosciences) were used for sequencing (Lee et al.,

178   2022a).

179   **SMRT sequencing**

180   SMRT sequencing was performed using a PacBio RSII system (Pacific Biosciences) according

181   to the manufacturer's instructions. In brief, 10-h movies were captured for each SMRT cell

182   (Pacific Biosciences). Subsequent steps were carried out based on the PacBio Sample Net-

183   Shared Protocol (https://www.pacb.com/). Circular consensus sequencing (CCS) reads, such as

184   raw sequence reads, were processed using the SMRT analysis software (version 2.3, Pacific

185   Biosciences). Short CCS reads and those with zero quality bases, considered as sequencing

186   errors, were removed (Lee et al., 2022a).

187   **Taxonomic and statistical analysis**

188   Taxonomic analysis of the CCS reads was performed using the MG-RAST server (Meyer et al.,

189   2008) with the SILVA SSU database (Quast et al., 2013). Then, in the pipeline version 4.0.3, the

190   pipeline options were set as follows: assembled, dereplication, screening, length filtering, length

191   filter deviation multiplicator, ambiguous base filtering, and maximum ambiguous base pairs

192   were set to 'yes,' 'no,' 'E. coli NCBI st. 536', '2.00', 'no', and '5', respectively. Moreover, the

193   e-value, percent identity, minimal alignment length, and minimal abundance values were set to

194   '5', '90', '15', and '1', respectively. Additionally, 'representative hit' was selected. Statistical

195   analyses were performed using MicrobiomeAnalyst (Dhariwal et al., 2017). Data normalization

196   for each sample was performed for total sum scaling. Abundance profiling was presented as a

197   stacked bar chart by calculating the percentage abundance, and less than 10 taxa were omitted.

198   Species richness based on the alpha diversity of samples was determined via rarefaction curve

199 analysis (McMurdie & Holmes, 2013), and the diversity of operational taxonomic units (OTUs)
200 was indicated by the Shannon index. In addition, the evenness of OTUs was indicated by the
201 pielou index (Jost, 2010). A heat tree was constructed using the non-parametric Wilcoxon rank
202 sum test and was used to statistically quantify the hierarchical structure of taxonomic
203 classification (Foster et al., 2017). The distance method and principal coordinate analysis
204 (PCoA) was used for determining the beta diversity of samples and were set to unweighted
205 UniFrac distance and permutational multivariate analysis of variance (PERMANOVA),
206 respectively.
207   In addition, the distance measure and clustering algorithm of the hierarchical clustering
208 algorithm (HCA) were set to the Bray–Cutis index and Ward, respectively. A heat tree was used
209 to compare and sum the microbial communities and ultramicrobial communities for each sample.
210 As a high-dimensional data analysis performed using a supervised machine learning algorithm,
211 random forest classification analysis was conducted to identify the variability of strains in
212 samples (Liaw & Wiener, 2002; Lee et al., 2022a).
213 **Transmission electron microscopy (TEM)**
214 For TEM observations via negative staining, droplets of the samples were mounted on a carbon
215 support film on a 150-mesh nickel grid, stained with 4% uranyl acetate for 10 min and 0.4% lead
216 citrate for 6 min, washed three times with deionized water, and air-dried. In addition, the samples
217 were prepared in the form of ultrathin sections. To this end, samples were fixed by the addition
218 of glutaraldehyde and paraformaldehyde adjusted to 2% in 0.05 M phosphate buffer (pH 7.2) and
219 then incubated at room temperature (15–25 °C) for 4.5 h under vacuum. The fixed samples were
220 washed three times for 15 min each with 0.05 M phosphate buffer at pH 7.2. The washed
221 samples were post-fixed with osmium tetroxide adjusted to 1% in 0.05 M phosphate buffer (pH
222 7.2) at room temperature for 1 h. The post-fixed samples were washed three times for 15 min
223 each with 0.05 M phosphate buffer at pH 7.2 and then dehydrated by passing through an ethanol
224 gradient from 50 to 100%. After dehydration, the samples were precipitated to resin (LR white
225 resin; EMS, Hatfield, PA, USA), placed in a disposable mold and embedded for 24 h at 60 °C.
226 After the sample was hardened, ultrathin sections were prepared using an ultramicrotome
227 equipped with a diamond knife and stained with 4% uranyl acetate for 10 min and 0.4% lead
228 citrate for 6 min to complete sample preparation for TEM observation. The prepared samples
229 were observed using a field-emission TEM (FE-TEM) (JEM-2100F; JEOL Ltd., Tokyo, Japan)
230 at 200 kV accelerating voltage (Lee et al., 2022a).
231 **Data availability**
232 The sequencing reads of fermented cabbages were deposited to the NCBI under BioProject ID
233 PRJNA684410, the metadata for each sample can be found in SRR13260135, SRR13260137,
234 SRR13260138, SRR13260156, SRR13260177, SRR13260178.
235
236 **Results**
237 **SMRT sequencing**

238   The NM and USM in fermented cabbage were separated using TFF and analyzed via SMRT
239   sequencing. SMRT sequencing read quality indices are shown in *Table S1*. Sequence read
240   information for each sample is presented in *Table 1*. A total of 19,356 sequence reads from
241   Kimchi_NM, 1,383 from Kimchi_USM, 1,208 from Sauerkraut_NM, 1,610 from
242   Sauerkraut_USM, 11,446 from Suancai_NM, and 1,570 from Suancai_USM were generated.
243   The total number of reads from NM was much greater than that of USM. Notably, the number of
244   reads from the Sauerkraut_USM was slightly higher than that from the Sauerkraut_NM.
245   Kimchi_NM had the greatest number of sequence bases at 28,690,199 bp, while Sauerkraut_NM
246   had the lowest at 1,741,058 bp. The average read lengths of the samples ranged from 1,425 to
247   1,482 bp, and were almost identical between groups (Lee et al., 2022a).
248   **Taxonomic and statistical analyses**
249   Community richness in the samples was expressed using the rarefaction curve (*Fig. 1A*), while
250   alpha diversity was expressed using the Shannon index (*Fig 1B*). Each sample in the rarefaction
251   curve plateaued. Suancai_NM had the highest richness (OTUs of 84), while Sauerkraut_NM had
252   the lowest (OTUs of 11). However, generally, the OTUs in Kimchi_NM and Suancai_NM were
253   more abundant than those in the Kimchi_USM, Sauerkraut_USM, and Suancai_USM, but not
254   than that in Sauerkraut_NM. Since the rarefaction curve of Kimchi_NM was gently inclined,
255   richness was high, while diversity was low. Based on the Shannon index, Kimchi_NM had the
256   lowest diversity at 0.26, while Kimchi_USM had 1.62. Comparing fermented cabbage types,
257   Sauerkraut_NM had a diversity of 1.45, Sauerkraut_USM had 1.13, Suancai_NM displayed the
258   highest diversity at 1.95, and Suancai_USM had 1.23. Suancai_NM had the highest microbial
259   diversity, whereas Kimchi_NM had the lowest. The alpha diversity of suancai was higher than
260   that of kimchi and sauerkraut when diversity was assessed between fermented cabbage types.
261   Further, USM had higher alpha diversity on an average when diversity was assessed based on
262   community type and regardless of the sample type. Based on the Pielou index, Kimchi_NM had
263   the lowest evenness at 0.03, while Kimchi_USM displayed the highest evenness at 0.43.
264   Comparing fermented cabbage types, Sauerkraut_NM had an evenness index of 0.31,
265   Sauerkraut_USM had 0.23, Suancai_NM had 0.26, and Suancai_USM had 0.26. In terms of
266   evenness among fermented cabbage types, sauerkraut and suancai showed relatively similar
267   values. While assessing evenness based on community type and regardless of sample type, USM
268   samples generally demonstrated higher evenness compared with NM samples, with the exception
269   of sauerkraut, where NM samples had higher evenness than USM samples (Lee et al., 2022a).
270   The NM and USM in kimchi and suancai are shown in *Fig 2A* and *Fig. S3*. At the phylum
271   level, Firmicutes were dominant in Kimchi_NM and Suancai_NM. In the former, Firmicutes
272   accounted for 100% of the microbial community. Actinobacteria dominated Kimchi_USM, and
273   uncultured bacteria dominated Sauerkraut_USM as well as Suancai_USM. At the species level,
274   *Weissella koreensis* was dominant at 94% in Kimchi_NM, while *Weissella cibaria* was also
275   present, but in minor quantities. *Cellulomonas uda* and *Cupriavidus pauculus* were predominant
276   in Sauerkraut_NM, at 32% and 26%, respectively. However, in Sauerkraut_NM, uncultured
277   bacteria accounted for a significant proportion (32%). *Lactobacillus acetotolerans* was dominant

278 (53%) in Suancai_NM. *Lactobacillus similis* was also predominant in Suancai_NM, accounting
279 for 11%. *Cellulomonas biazotea* dominated at 42% in Kimchi_USM. In particular, candidate
280 division TM7 single-cell isolate TM7a (TM7a), known as *Saccharibacteria*, was predominant at
281 35%. *Cellulomonas uda* predominated in Sauerkraut_USM at 36%. However, uncultured soil
282 bacteria dominated Sauerkraut_USM at 54% and Suancai_USM at 48% (Lee et al., 2022a).
283   The OTU values indicated that the species richness of NM is generally higher than that of
284 USM (*Fig. 1*). In particular, Suancai_NM exhibited the highest abundance and alpha diversity, as
285 indicated by significantly higher OTU and Shannon index values of the microbiome compared to
286 that of the other samples. However, the Pielou index value of NM was 0.26, which was relatively
287 low compared with other samples, and was the same as that of USM. Therefore, Suancai's USM
288 and NM were almost equal in evenness, and evenness was inferior to that of Sauerkraut_NM
289 with a Pielou index value of 0.31. For Kimchi_NM, the OTU value was relatively higher than
290 that for other samples, however, its Shannon index value was the lowest. In addition, the
291 rarefaction curve of Kimchi_NM only slightly increased, suggesting low diversity. The Shannon
292 index was lower than the OTU value because *Weissella koreensis* showed > 94% dominance. In
293 contrast, *L. acetotolerans* showed > 53% dominance in Suancai_NM, while other species
294 showed minor dominance, between 1 and 11%. Therefore, the Shannon index of Suancai_NM
295 was estimated to be the highest. In addition, The OTU values of USM were relatively low, with
296 the one for Kimchi_USM being the lowest. However, Shannon index of Kimchi_USM was the
297 second highest, > 1.6, possibly due to the even distribution of species. As proof of this, the
298 Pielou index value of Kimchi_USM was 0.43, indicating higher evenness than other samples.
299 While the difference in alpha diversity values between the NM and USM of kimchi and suancai
300 was high, the difference in alpha diversity value between the NM and USM of sauerkraut was
301 small. Since sterilization was performed during the manufacturing process of sauerkraut, most of
302 the normal microorganisms did not survive. Therefore, the difference in alpha diversity between
303 Sauerkraut_NM and Sauerkraut_USM was not expected to be high. The species richness of USM
304 was low, yet the diversity was higher than that of NM. The microbial distribution of the fraction
305 that passed through the 0.2-μm filter, which is sterile, was more diverse than expected (Nakai,
306 2020).
307   The beta diversity of NM and USM in fermented cabbage samples was compared via
308 PCoA (*Fig. 2B*) and HCA (*Fig. 2C*). The plot and dendrogram showed that Suancai_NM and
309 Kimchi_USM were closely related as also observed for Sauerkraut_NM, Sauerkraut_USM, and
310 Suancai_USM. In addition, PCoA indicated that Kimchi_NM was separated from the other
311 samples. However, Suancai_NM, Kimchi_USM, and Kimchi_NM were grouped in the HCA
312 dendrogram, as were Suancai_USM, Sauerkraut_NM, and Sauerkraut_USM (Lee et al., 2022a).
313   A heat tree was used to compare and sum the NM and USM at the genus level for each
314 fermented cabbage (*Fig. 3*). The sum of NM and USM per fermented cabbage type is shown in
315 *Fig. 3A–C*. Obtaining this sum was equivalent to combining the NM and USM of each fermented
316 cabbage in the bar chart of *Fig. 2A*. *Figures 3D–F* compare the NM and USM in each fermented
317 cabbage. In kimchi, several genera belonging to the phylum Firmicutes had a relatively high ratio

318 in the NM compared to the USM (*Fig. 3D*). Unlike in kimchi, in sauerkraut, several genera
319 belonging to the phylum Proteobacteria had a relatively higher ratio in the NM compared to that
320 in the USM (*Fig. 3E*). Suancai was similar to kimchi as several genera belonging to the phylum
321 Firmicutes had a relatively higher ratio in the NM compared to that in the USM (*Fig. 3F*) (Lee et
322 al., 2022a).
323   The random forest algorithm was used to confirm the top 15 OTUs with large variability
324 between the microbiomes (*Fig. 4*). The mean reduced accuracy represents the accuracy that the
325 microbiome loses by excluding each variable. Thus, the lower the accuracy, the more important
326 the variable species is for a successful classification. Species are displayed in descending order
327 of importance, that is, the higher the mean reduction accuracy, the higher the importance of the
328 species in the microbiome (Martinez-Taboada and Redondo, 2020). Since the mean decrease
329 accuracy of uncultured bacteria was the highest (0.0279), the microbiome was likely to be
330 divided based on the content of these strains. The mean decrease in accuracy of uncultured
331 *Azospira* sp. and *Lactobacillus paracollinoides* was 0.0231 and 0.0230, respectively, which were
332 the second and third highest, respectively (Lee et al., 2022a).
333 **Morphological observations via TEM**
334 TEM was used to confirm the presence of microorganisms in the USM of fermented cabbage
335 samples (*Fig. 5* and *Fig. S4*). Most of the USM were cocci with both outer and inner membranes
336 observed in the USM isolated from all fermented cabbages. In addition, the periplasmic space
337 between the outer and inner membranes was observed (*Fig. 5C, G*, and *K*). The size of the
338 microorganism in USM was approximately 100–200 nm. In addition, USM isolated from
339 fermented cabbages had multiplied via dichotomy (*Fig. 5B* and S4H, K Fig) (Lee et al., 2022a).
340
341 **Discussion**
342 In the present study, we identified the NM and USM in different fermented cabbages via TFF
343 and SMRT to characterize and compare USM between cabbage types. TFF has been used to
344 concentrate various microorganisms in water and is an excellent technique for their separation or
345 removal (Cai et al., 2015). TFF has shown 11–98% recovery for plankton viruses, smaller than
346 0.2 μm in size, from freshwater samples (Colombet et al., 2007). Therefore, in the present study,
347 we employed TFF instead of conventional normal flow filtration (NFF) to separate and
348 concentrate NM and USM from kimchi, sauerkraut, and suancai. TFF is more efficient than NFF
349 since it can filter more liquid phase by smoothly removing the filter cake. In addition, while there
350 were fewer sequence reads for USM than those for NM (*Table 1*), they might not have been
351 obtained if not enriched via TFF (Lee et al., 2022a).
352   SMRT sequencing, a synthetic long-read sequencing technology based on full-length 16S
353 rRNA, is widely used to study microbial communities in various environments, including food. It
354 has been used to analyze microbial diversity in various samples (Pootakham et al., 2017; Jeong
355 et al., 2021; Hui et al., 2022; Liang et al., 2022). SMRT sequencing is advantageous over general
356 Illumina sequencing because it provides accurate classification at a taxonomic level using full-
357 length 16S rRNA sequences instead of short amplicons (Mosher et al., 2014; Jeong et al., 2021);

358   in this study, SMRT sequencing was used to confirm the microbial composition of fermented
359   cabbage, including NM and USM samples.
360   Moreover, we categorized the sequences as Operational Taxonomic Units (OTUs).
361   Indeed, recent studies suggest that Amplicon Sequence Variants (ASVs) offer a higher
362   taxonomic resolution than OTUs, and additionally correct sequencing errors, thereby providing a
363   more accurate representation of microbial diversity (Callahan et al., 2017; Nearing et al., 2018;
364   Chiarello et al., 2022). However, it's worth noting that no significant difference has been found
365   between the results yielded by analyses of OTUs and ASVs (Glassman and Martiny, 2018).
366   OTUs have long served as a simple and intuitive method of classification, and due to their
367   widespread use over the years, a greater number of studies employ OTUs compared to ASVs.
368   This prevalence allows for a more comprehensive comparison of our research with existing
369   literature.
370   *Weissella koreensis*, a dominant bacterium in kimchi (Jung et al., 2014), was first
371   reported in kimchi in 2002 (Lee et al., 2002). Further, *Weissella* spp., including *W. koreensis*, are
372   involved in kimchi fermentation (Cho et al., 2006). In line with previous reports, this study also
373   showed that *W. koreensis* was highly prevalent in Kimchi_NM. However, in Kimchi_USM, *C.
374   biazotea*, which is known to degrade cellulose (Rajoka & Malik, 1997), and TM7a, which is
375   known to be parasitic on bacterial hosts (Marcy et al., 2007; Bor et al., 2019), were both detected
376   (Lee et al., 2022a).
377   The microbial abundance of USM and NM in each sample was relatively normalized via
378   total sum scaling, however, because the number of sequence reads from Kimchi_USM was over
379   ten times less than that of Kimchi_NM, *C. biazotea* and TM7a do not represent the microbiome
380   in general kimchi containing USM and NM. In addition, based on the sequence reads, the ~~present~~
381   ratio of USM to NM in kimchi was 7.1, which was the basis for the lower microbial abundance
382   in USM compared with NM. Further, if the USM was not separated via TFF, *C. biazotea* and
383   TM7a would not have been identified in kimchi. We reviewed the available literature on the
384   microbiome of kimchi and found no reports on *C. biazotea* and TM7a in kimchi via culture-
385   dependent or culture-independent methods (Cho et al., 2006; Park et al., 2012; Jung et al., 2014;
386   Lee et al., 2017; Maoloni et al., 2020; Park et al., 2020; Lee et al., 2022a).
387   The 16S rRNA gene is shared among all bacteria and utilizing this gene would
388   significantly reduce the labor and cost of profiling the identity and abundance of microorganisms
389   in various environments, regardless of the culture capacity (Hugenholtz et al., 2021). However,
390   the 16S rRNA gene is not an optimal target, owing to the short read length of most commonly
391   used sequencing platforms, such as Illumina, which limits the taxonomic resolution to families or
392   genera (Earl et al., 2018; Jeong et al., 2021). However, if the 16S rRNA gene is almost entirely
393   sequenced using SMRT sequencing, which can also analyze long sequences, taxonomic
394   resolution can be improved. In a previous study, 60% of specific phyla, such as the phylum
395   Microgenomates*,* were not detected via PCR using the 518F and 806R primer sets (Brown et al.,
396   2016), and the low taxonomic resolution due to short sequencing limits the amount of data on
397   microbial ecology (Lee et al., 2022a). Indeed, SMRT sequencing offers a significant advantage

398   over Illumina sequencing in terms of taxonomic resolution because it covers the entire variable
399   region (V1–V9) of the 16S rRNA gene (Mosher et al., 2014). In contrast, Illumina sequencing
400   typically covers only a narrow region. This increased resolution allows for accurate identification
401   and classification of microbial taxa, particularly for rare or hard-to-detect species, which may not
402   be as effectively characterized using short-read sequencing platforms, such as Illumina.
403   Therefore, the identification of *C. biazotea* and TM7a in kimchi may be attributed to the use of
404   SMRT sequencing in the current study. In particular, TM7, also known as *Saccharibacteria*,
405   have been reported in the oral cavity (Bor et al., 2019) and are known to be ultra-small (200–300
406   nm) and parasitic bacteria attached to the surface of host bacteria (Bor et al., 2016), which
407   complicates their detection via conventional culture methods. Further, TM7 does not grow unless
408   a special method of symbiosis is employed (Murugkar et al., 2020). The causal relationship
409   observed herein remains unclear, and further studies are warranted to determine how TM7 is
410   transmitted to humans. In addition to *C. biazotea* and TM7a, other bacteria were rarely found in
411   Kimchi_USM. Therefore, most of the bacteria in the USM did not adapt to the kimchi
412   environment or lost their survival competition to *Weissella* spp. (Lee et al., 2022a).
413   Sauerkraut_NM and Sauerkraut_USM were dominated by *C. uda*. Like *C. biazotea, C.
414   uda* secretes cellulases. The genus *Cellulomonas* is abundant in soil (Robinson, 2014) and was
415   found in Sauerkraut_NM and Sauerkraut_USM, probably derived from the soil where the raw
416   cabbage had been planted. Furthermore, while the genus *Cellulomonas* is relatively rare, it may
417   potentially be pathogenic because there have been instances of human infection (Salas et al.,
418   2014). Martin et al. discovered novel USM from the class *Actinobacteria,* belonging to the genus
419   *Cellulomonas*, in five freshwater reservoirs (Hahn et al., 2003). Although only Actinobacteria
420   living in freshwater environments were mentioned, *Cellulomonas* in soil should also have several
421   USM types. *Cupriavidus pauculus* (also known as *Ralstonia paucula*), detected only in
422   Sauerkraut_NM, can pass ultrafiltration (Cuadrado et al., 2010), although it was not found in
423   Sauerkraut_USM. In addition, *C. pauculus* may not occur in a USM, because it is a filterable
424   bacterium (Lee et al., 2022a).
425   Suancai_NM was dominated by *L. acetotolerans*, which was first reported in fermented
426   vinegar broth (Entani et al., 1986). *Lactobacillus* spp. are involved in suancai fermentation (Liu
427   et al., 2019). However, *L. acetotolerans* was previously reported as abundant in pao cai, but not
428   in suancai (Cao et al., 2017; Liu et al., 2019). These differences are likely attributable to
429   variations in the manufacturing processes (e.g., temperature, salinity, and seasoning), and these
430   distinctions can be observed in *Fig. S1*. Uncultured bacteria, including soil bacteria, were
431   dominant in Suancai_USM, and these OTUs may not be included in the SILVA SSU database.
432   Similarly, in Sauerkraut_NM and Sauerkraut_USM, uncultured soil bacteria and uncultured
433   bacteria were also dominant, while random forest analysis indicated that their OTUs exhibited
434   large variation among samples (*Fig. 4*) (Lee et al., 2022a).
435   *Ralstonia* spp. were detected in both Sauerkraut_USM and Suancai_USM at < 1%.
436   However, *Ralstonia* spp., similar to *C. pauculus*, are filterable bacteria, hence, they are classified
437   as USM but are most likely not UMB. In addition, since they can survive by attaching to the

438 ultrapure water system (Kulakov et al., 2002), they may not be resident microorganisms in
439 kimchi or suancai. Detected at < 1%, *Ralstonia* spp. do not present a major bias. Nevertheless,
440 some species within these genera are pathogens, and may potentially serve as pathogenic agents;
441 a thorough sterilization may be necessary when similar studies are conducted in the future.
442   Comparison of the microbial and ultramicrobial communities of kimchi, sauerkraut, and
443 suancai via PCoA and HCA (Fig 2B and 2C) showed differences between them. In addition,
444 although each fermented cabbage had distinct microbial community (*Fig. 3A–C*), the heat tree
445 indicated differences between sample NM and USM (*Fig. 3D–F*). While the main ingredients
446 (*Brassica rapa* subsp. *pekinensis*) of kimchi and suancai are similar, differences in the
447 manufacturing method, other ingredients, seasoning, and the surrounding environment might
448 have contributed to the prevalence of different microbial and ultramicrobial communities. Since
449 sauerkraut was sterilized in the manufacturing process, the difference between the microbial and
450 ultramicrobial communities was not high and was very similar to Suancai_USM. In sauerkraut,
451 the genes of both NM and USM were mixed with those of dead cells due to sterilization; the
452 reason behind the microbial abundance not being high, even though the genes of the dead cells
453 were mixed, is presumed to be that the genome of the dead bacteria was decomposed by the high
454 temperature during the sterilization process (Eichmiller et al., 2016; Tsuji et al., 2017; Lee et al.,
455 2022a).
456   Although we successfully identified USM between 0.2 μm and 100 kDa in fermented
457 vegetables, TFF and SMRT sequencing methodologies may lead to misrecognition of fragments
458 of bacteria (Duda et al., 2011). Therefore, we sought to confirm the presence of ultra-small
459 microorganisms in USM via TEM (Fig 5 and S4 Fig). The ultra-small microorganism in
460 Kimchi_USM, Sauerkraut_USM, and Suancai_USM were observed as coccoid types, 100–200
461 nm in size, with outer and inner membranes, as well as multiplication via dichotomy; therefore,
462 the existence of ultra-small microorganism in the UMB of fermented cabbages was confirmed.
463 The possibility that it is an outer membrane vesicle cannot be excluded (Cecil et al., 2019).
464 However, bisection was observed; therefore, the possibility that it is an ultra-small
465 microorganism cannot be ruled out (Lee et al., 2022a).
466   Rod shape bacteria such as genera *Cellulomonas*, *Cupriavidus*, and *Lactobacillus* were
467 also detected in USM using SMRT, while only spherical shape bacteria were found in TEM.
468 Since, as previously suggested, spherical shaped bacteria must be at least 250–300 nm in
469 diameter to maintain the 250–300 proteins essential for life, the normal state may therefore be
470 rod shape; however, it might have transformed into a spherical shape for survival (Ghuneim et
471 al., 2018). Moreover, USM formation, even in nutrient-rich environments, was possibly
472 attributed to the action of predators and selective pressures such as pH and drying (Simon et al.,
473 2002; Pernthaler, 2017; Lee et al., 2022a).
474   In NM or USM extracted from fermented cabbages, major human pathogenic bacteria
475 were not detected. However, although the number of OTUs and sequence reads were small,
476 several taxonomic groups suspected of causing human diseases, such as *Ralstonia* were detected
477 in USM, and a significant proportion of uncultured bacteria with or without pathogenicity were

478 also detected. Although these bacteria were smaller than the general pore size of the sterilizing
479 filter, which is 0.2 µm, and may not be harmful immediately, there is a concern that there may be
480 potential risks when the fermented cabbages are further processed into sauces, juices, etc.
481 Furthermore, based on a previous study (Lee et al., 2022b), it was suspected that USM may be in
482 a VBNC state. Although USM was not starving due to the abundant nutrients in the fermented
483 cabbages, it is possible that the dominance of lactic acid bacteria due to fermentation could have
484 led to stress and difficulty in growth, resulting in conversion to ultra-small microorganisms in a
485 VBNC state. VBNC bacteria can survive under severe stress conditions, such as starvation, low-
486 temperature sterilization, and antibiotics, and some subgroups can survive without time-
487 dependent changes in gene expression as a response to stressful stimuli (Chaveerach et al., 2003;
488 Li et al., 2014; Ramamurthy et al., 2014; Ayrapetyan & Oliver, 2016). In addition, VBNC
489 bacteria, similar to persistent bacteria, probabilistically can exist even in rapidly growing
490 environments (Ayrapetyan et al., 2015; Goncalves & de Carvalho, 2016; Orman et al., 2016).
491 Therefore, the transition to a VBNC state is an important issue in food hygiene (Dong et al.,
492 2020), and USM should also be considered as having potential risks. In addition, Li and
493 colleagues reported that the use of acetic acid can inhibit the VBNC state of *E. coli* O157:H7 (Li
494 et al., 2020). Thus, it is possible to reduce the potential risks of USM suspected of being in a
495 VBNC state by adjusting the concentration of organic acids or using other methods.
496   Although this study was conducted rigorously, there remains the possibility of
497 contamination from external microbial DNA during the sampling or DNA extraction process.
498 Moreover, it may be challenging to consider the samples in this study as fully representative of
499 all fermented cabbage varieties. However, this study holds significance because it is the first to
500 investigate USM in nutrient-rich foods such as fermented cabbages, shedding light on the
501 potential risks associated with their presence. Therefore, we believe that this study will serve as a
502 solid foundation for future studies on fermented foods and USM fostering further advancements
503 in the field.

504 **Conclusions**
505 In conclusion, our findings revealed that USM in cabbage differed from NM, and although major
506 pathogens were not detected within USM, several potentially concerning strains, such as those
507 from the genera *Cellulomonas* and *Ralstonia*, were detected. This suggests that poor sanitization
508 in the manufacturing environment could lead to the presence of ultra-small microorganisms in
509 fermented food products, posing a risk to consumer health. Overall, our study offers new insights
510 into the USM in food, specifically in fermented cabbage, and underscores the importance of
511 proper sanitization during production.

512

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515

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774

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).
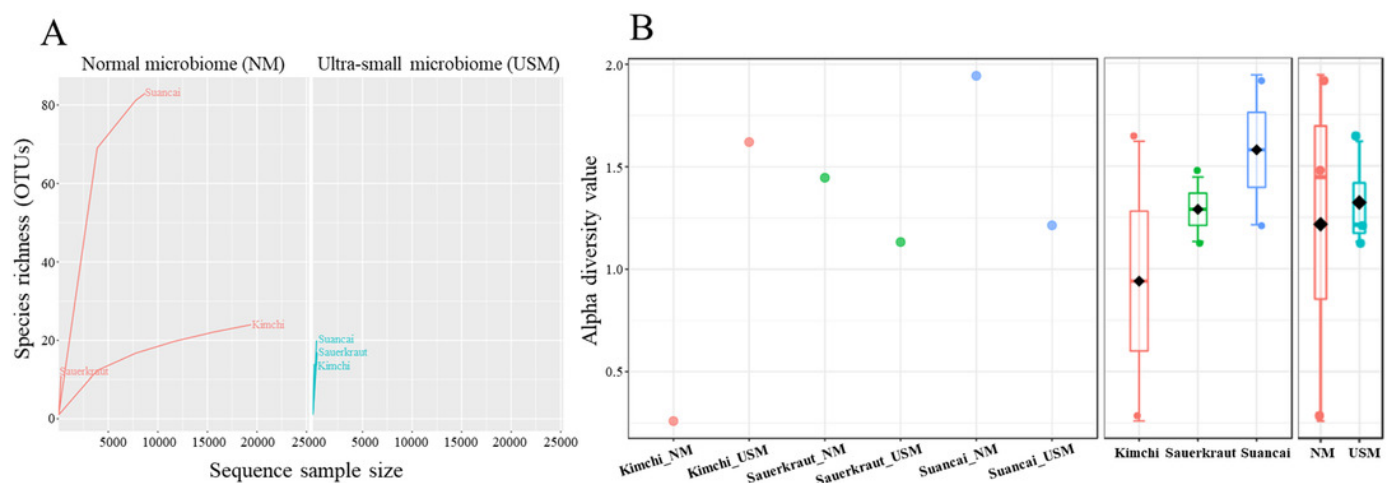
Figure 2

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.
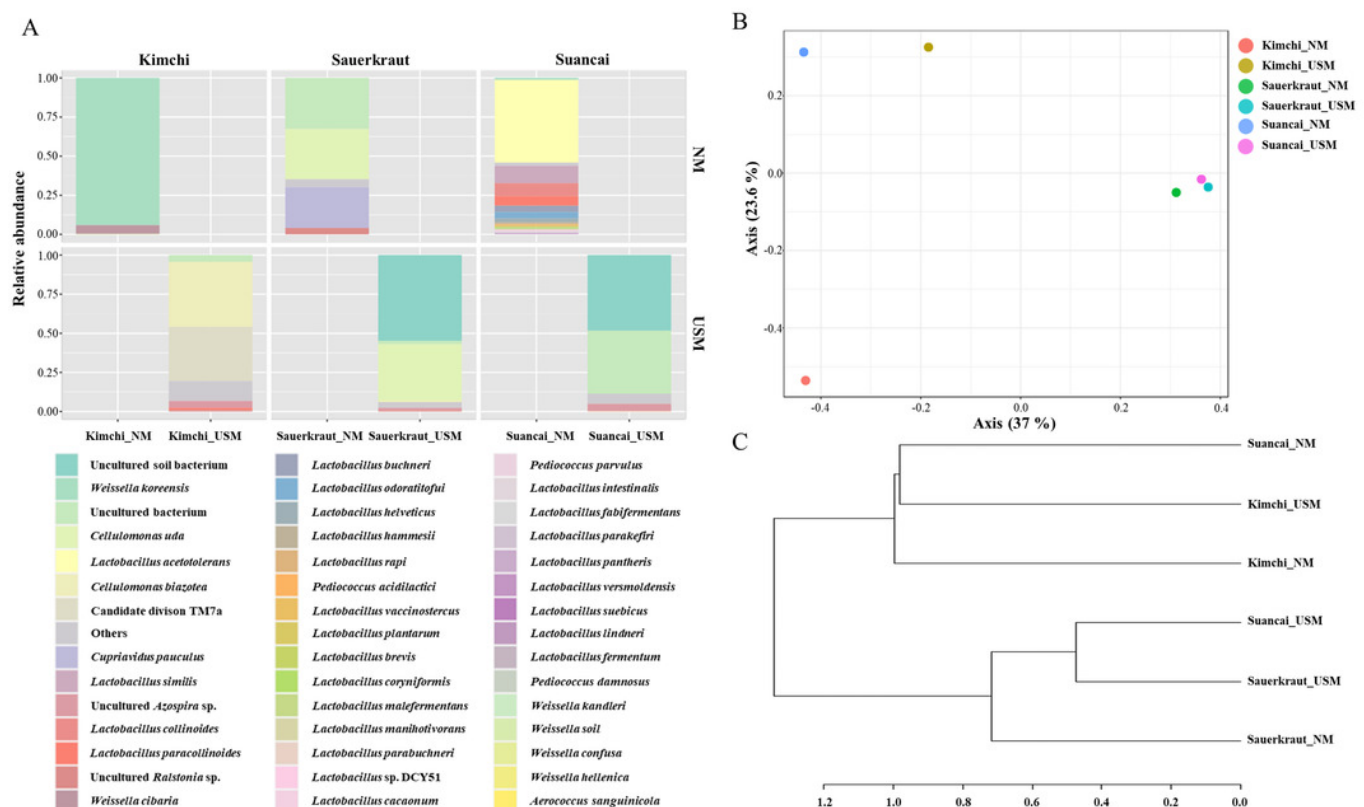
Figure 3

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.
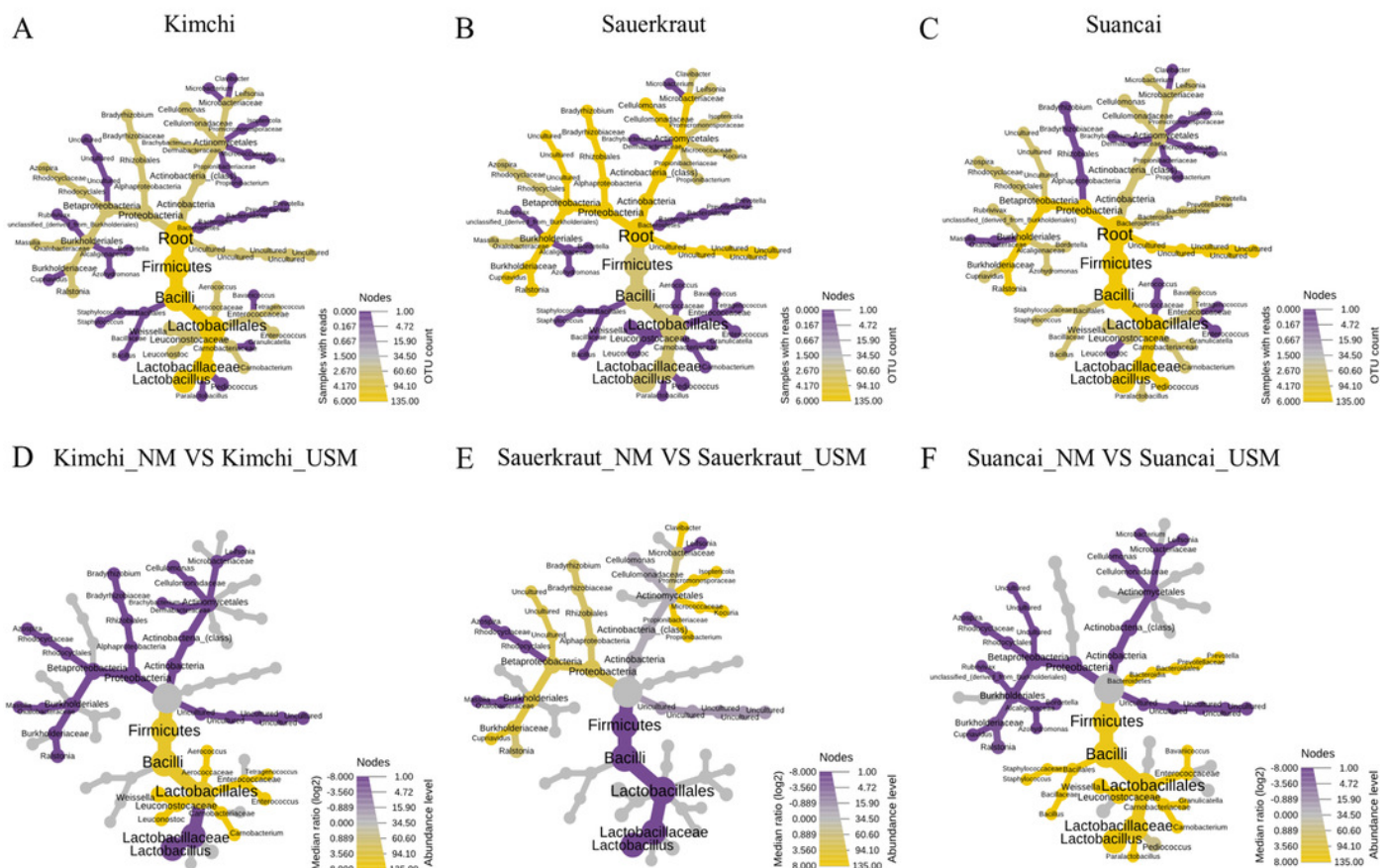
Figure 4

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.
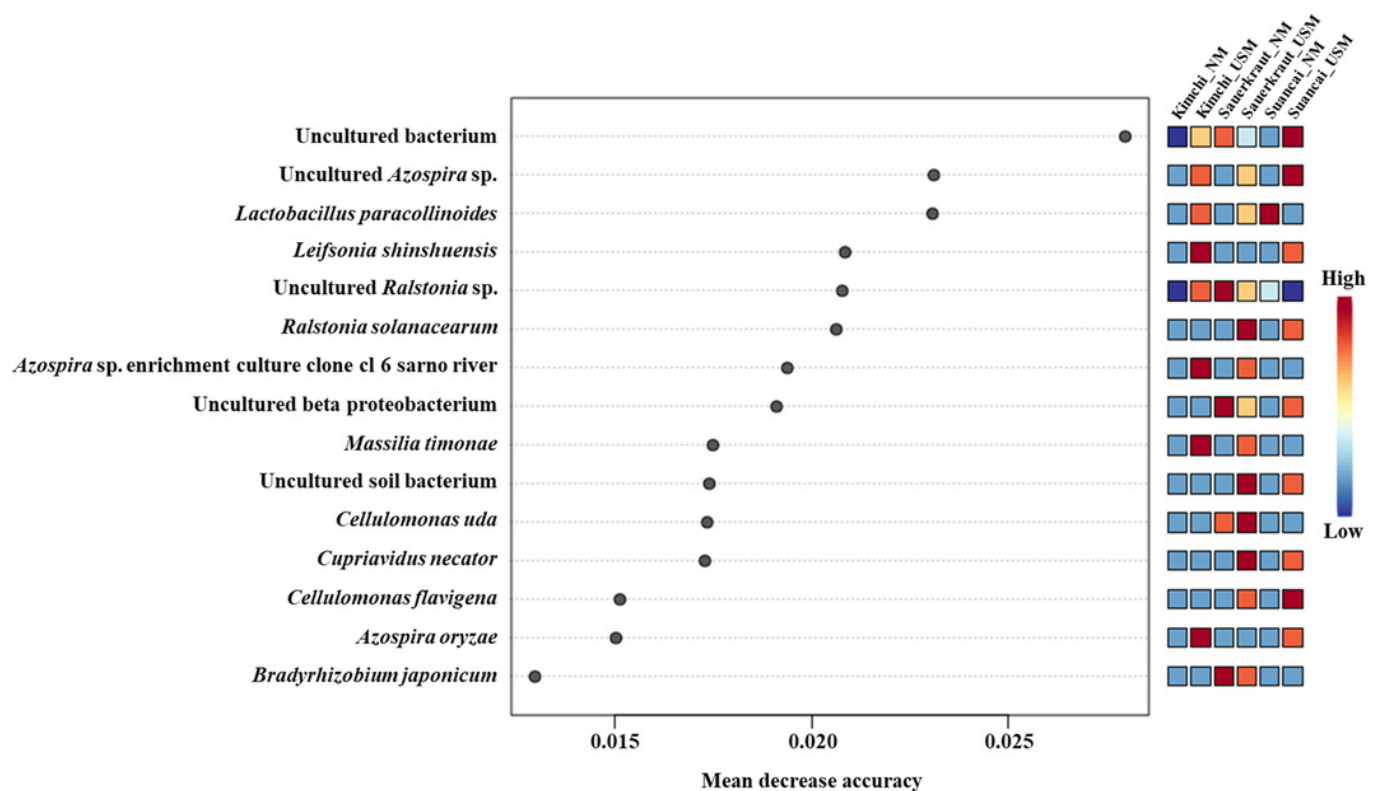
Figure 5

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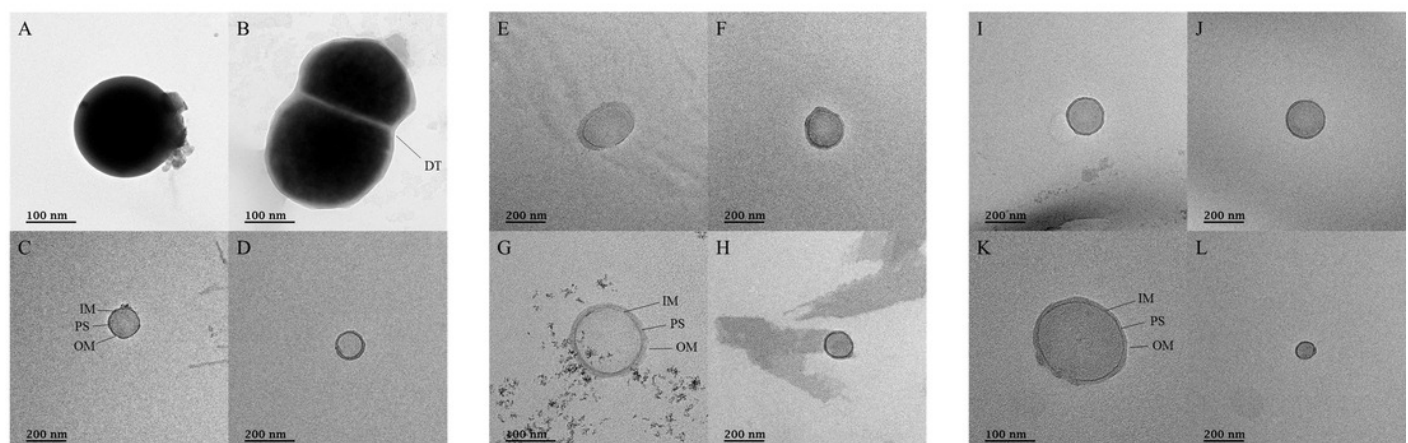
Table 1. States of sequence reads for each sample after trimming.

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.

1 **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 ± 278[a]
Kimchi_USM	1,383	1,983,758	1,434 ± 271
Sauerkraut_NM	1,208	1,741,058	1,441 ± 288
Sauerkraut_USM	1,610	2,293,503	1,482 ± 258
Suancai_NM	11,446	16,750,589	1,463 ± 296
Suancai_USM	1,570	2,237,604	1,425 ± 246

2 Kimchi_NM, normal microbiome > 0.2 μm in size from kimchi; Kimchi_USM, ultra-small
3 microbiome < 0.2 μm in size from kimchi; Sauerfkraut_NM, normal microbiome > 0.2 μm in
4 size from sauerkraut; Sauerkraut_USM, ultra-small microbiome < 0.2 μm in size from
5 sauerkraut; Suancai_NM, normal microbiome > 0.2 μm in size from suancai; Suancai_USM,
6 ultra-small microbiome < 0.2 μm in size from suancai.
7 [a], standard deviation for the average read length
8