Metatranscriptomic and metataxonomic insights into the ultra-small microbiome of the Korean fermented vegetable, kimchi

Hae-Won Lee¹,²,³, So-Ra Yoon¹, Yun-Mi Dang¹, Ji-Hyun Yun²,³, Hoibin Jeong⁴,⁵, Kil-Nam Kim⁴, Jin-Woo Bae²,³* and Ji-Hyoung Ha*¹

¹Hygienic Safety Packaging Research Group, World Institute of Kimchi, Gwangju, South Korea; ²Department of Biology, Kyung Hee University, Seoul, South Korea; ³Department of Life and Nanopharmaceutical Sciences, Kyung Hee University, Seoul, South Korea; ⁴Chuncheon Center, Korea Basic Science Institute (KBSI), Chuncheon, South Korea; ⁵Korea Research Institute of Bioscience and Biotechnology (KIRIBB), Daejeon, South Korea

Presently, pertinent information on the ultra-small microbiome (USM) in fermented vegetables is still lacking. This study analyzed the metatranscriptome and metataxonome for the USM of kimchi. Tangential flow filtration was used to obtain a USM with a size of 0.2 μm or less from kimchi. The microbial diversity in the USM was compared with that of the normal microbiome (NM). Alpha diversity was higher in the USM than in NM, and the diversity of bacterial members of the NM was higher than that of the USM. At the phylum level, both USM and NM were dominated by Firmicutes. At the genus level, the USM and NM were dominated by Lactobacillus, Leuconostoc, and Weissella, belonging to lactic acid bacteria. However, as alpha diversity is higher in the USM than in NM, and the diversity of bacterial members of the NM was higher than that of the USM. At the phylum level, both USM and NM were dominated by Firmicutes. At the genus level, the USM and NM were dominated by Lactobacillus, Leuconostoc, and Weissella, belonging to lactic acid bacteria. However, as alpha diversity is higher in the USM than in the NM, the genus Akkermansia, belonging to the phylum Verrucomicrobia, was detected only in the USM. Compared to the NM, the USM showed a relatively higher ratio of transcripts related to “protein metabolism,” and the USM was suspected to be involved with the viable-but-nonculturable (VBNC) state. When comparing the sub-transcripts related to the “cell wall and capsule” of USM and NM, USM showed a proportion of transcripts suspected of being VBNC. In addition, the RNA virome was also identified, and both the USM and NM were confirmed to be dominated by pepper mild mottle virus (PMMoV). Additionally, the correlation between metataxonome and metatranscriptome identified USM and NM was estimated, however, only limited correlations between metataxonome and metatranscriptome were estimated. This study provided insights into the relationship between the potential metabolic activities of the USM of kimchi and the NM.

KEYWORDS
metatranscriptome, metataxonome, tangential flow filtration, ultra-small microbiome, kimchi
Introduction

Kimchi is not only a representative food of Korea (Kim et al., 2016) but is one that has been consumed in Korea for the past 2000 years (Kwon et al., 2014; Park et al., 2014). Kimchi, of which there are approximately 200 types is a salted fermented vegetable made by first salting kimchi cabbage (Brassica rapa subsp. pekinensis), mixing it with condiments such as red pepper powder, onion, garlic, and ginger, and then allowing it to ferment. Kimchi is, therefore, considered unique in its manufacturing method compared to other fermented-salted vegetables that are primarily pickled in salt or vinegar. Further, kimchi has been reported to be a good source of probiotics containing lactic acid bacteria (Song and Lee, 2014) and has been suggested as a functional food with low calories that is rich in vitamins and minerals as well as dietary fiber (Kim et al., 2016). Recently, during the coronavirus pandemic, the antiviral effects of kimchi became known (Bousquet et al., 2021; Das et al., 2021), and kimchi, which was mainly consumed in East Asia, is now being consumed worldwide (Lee and Ko, 2021).

The shotgun-based high throughput sequencing approach provides an opportunity to map the microbiome of foods to an unprecedented depth, highlighting the importance and key factors shaping the composition and activity of the resident microbiome, influencing food quality and safety (De Filippis et al., 2021; Quijada et al., 2022). An example is metatranscriptome analysis, which interprets RNA transcripts by high-throughput sequencing and offers unprecedented opportunities to analyze the functional and taxonomic dynamics of a microbiome (Bikel et al., 2015; Gallardo-Becerra et al., 2020).

Since ultramicrobacteria (UMB) were first defined by Torrella and Morita (1981), they have been identified in various environments such as soil, sand, ice, lake water, and seawater (Nakai, 2020), and an ultra-small microbiome (USM) containing UMB has been identified (Cai et al., 2015; Ghuneim et al., 2018; Proctor et al., 2018; Liu et al., 2019). In this study, USM is a microbiome containing ultra-small size microorganisms such as bacteria, endospores, and filterable bacteria, whose size has been reduced by external extreme environmental conditions, as well as UMB. The USM has been defined fairly comprehensively as a microbiome that passed through a 0.2 μm pore size filter.

In our previous study (Lee et al., 2022), although there was little possibility of expression, traces of pathogens remained in USM. It was thought to be possible that judging from this, some bacteria such as VBNC, persisters, spore forms, and filterable forms in USM may act as potential microbiological hazards in fermented foods, whereas, if there are previously unknown health benefits of USM, it may be possible to make a unique product by fractionating and concentrating it. Although this USM may be important in food, little research on it is currently being done. Moreover, as far as we know, no metatranscriptome analysis has been attempted on the USM of fermented foods.

Our group recently confirmed the presence of communities of UMB of size 0.2 μm or less in fermented cabbages such as kimchi and sauerkraut using tangential flow filtration (TFF) and 16S rRNA gene amplicon sequencing (Lee et al., 2022). It was also revealed that the diversity of ultra-small bacterial microbiomes smaller than 0.2 μm in fermented cabbages was high. However, that study could not confirm the bacterial microbiome and metabolic activities. Therefore, in this study, we analyzed the metatranscriptome and metataxonome for the USM of kimchi. We also tried to gain insights into the relationship between the potential metabolic activities of the USM of kimchi and the microbiome.

Materials and methods

Sample preparation

For this study, kimchi made in South Korea was purchased from an online market in October 2021. The main ingredient of kimchi is kimchi cabbage (Brassica rapa subsp. pekinensis), and 10 packets of kimchi of 400 g each (to make handling easier) were purchased. After purchase, the pH of kimchi was measured with an automatic pH/mV titrator (TitroLine 5000, SI Analytics, Germany). All the kimchi samples were merged in a double-structured basket sterilized with a built-in strainer, and broths from merged kimchi samples were obtained and roughly filtered by the strainer. Then, 500 ml of Dulbecco's phosphate buffered saline (DPBS, Welgene, Korea) was added to the basket to facilitate filtration. The work on sample preparation was done inside a clean bench.

Pre-filtration and tangential flow filtration

Pre-filtration and TFF were performed similarly to the previous study (Lee et al., 2022). The broth obtained from the kimchi samples was pre-filtered by a polypropylene capsule filter (GVS Filter Technology, United States) with a pore size of 10 μm for TFF. Pre-filtration was carried out smoothly using a vacuum pump inside a clean bench.

Pre-filtered kimchi broth was injected into a TFF system (Cogent μScale TFF System, Millipore, United States), and TFF was performed in two phases. In the first phase, a 0.22 μm pore size TFF cartridge (Pellicon 2 Mini Cassette, Media: Durapore 0.22 μm, Millipore, United States) was used to confirm a normal microbiome (NM) larger than 0.2 μm in size. Then the NM was concentrated to 20 ml, and after dividing by each 10 ml, 25 ml of RNAlater (Sigma, United States) for metatranscriptomic analysis was separately added to 10 ml of concentrate for stabilization and protection of RNA. The remaining 10 ml was used for metataxonomic analysis. The prepared NM samples were stored in a −20°C freezer until analysis. A 100kDa molecular weight cut-off (MWCO) TFF cartridge (Pellicon 2 Mini Cassette, Media: Biomax 100kDa, Millipore, United States) was used in the second phase to confirm a USM smaller than 0.2 μm in size. Then the
USM was concentrated to 20 ml, and after dividing by each 10 ml, 25 ml of RNA later (Sigma, United States) for metatranscriptomic analysis was separately added to 10 ml of concentrate for stabilization and protection of RNA. The remaining 10 ml was used for metataxonomic analysis. The prepared USM samples were stored in a −20°C freezer until analysis.

**Metataxonomic analysis**

For metataxonomic analysis based on the 16S rRNA gene, DNAs were extracted using a DNeasy PowerSoil kit (Qiagen, Germany) for each sample solution of NM and USM. The extracted DNAs were quantified using the Quant-IT PicoGreen assay kit (Invitrogen, United Kingdom) following the manufacturer’s instructions. The quantified nucleic acids were amplified for the V3 and V4 regions of the 16S rRNA gene by a universal primer pair with Illumina adapters (V3-F, 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTATCGGNGGCCWGCAC-3′; V4-R, 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGACAGAGACACTACHVGGGTATCTAACTCC-3′). The polymerase chain reaction (PCR) conditions were as follows: initial denaturation at 94°C for 3 min, followed by 25 cycles of denaturation at 95°C for 30 s, then annealing at 55°C for 30 s, then extension at 72°C for 30 s, and a final extension at 72°C for 5 min. The PCR products were purified using AMPure beads (Agencourt Bioscience, United States). The purified PCR products were re-amplified to 15 cycles, including initial denaturation and final extension steps, as described with the Nextera XT indexed primer pair. The re-amplified PCR products were re-purified using AMPure beads and were quantified using the KAPA Library Quantification kits for Illumina sequencing platforms (Kapa Biosystems, United States). The qualification was carried out using the TapeStation D1000 ScreenTape (Agilent Technologies, United States). The paired-end (2 × 300 bp) sequencing was performed using the NovaSeq platform (Illumina, United States).

The annotation for the metatranscriptome of USM and NM was carried out by the MG-RAST server, and the paired-end sequence read files were also merged and trimmed by the server. Viromic analysis was performed using the ReSeq database (Pruitt et al., 2007), and the hierarchical functional analysis was performed using the SEED subsystem database (Overbeek et al., 2005).

**Statistics and visualization**

Statistical analyses for the metataxonome and metatranscriptome of USM and NM were done using MicrobiomeAnalyst (Dhariwal et al., 2017). The data normalization was performed for the total sum scaling. The alpha diversities of the metataxonome were calculated using the Phyloseq package (McMurdie and Holmes, 2013) with MicrobiomeAnalyst. A heat map was made using MultiExperiment Viewer 4.9.1 (Saeed et al., 2003), and Venn diagrams were created using VENNY 2.1.1. Correlation analysis between the metataxonome and metatranscriptome was performed using metaboAnalyst 5.0 (Pang et al., 2021), then bootstrapping for re-sampling was performed 20 times using XLSTAT 2017 (Addinsoft, France).

**Results and discussion**

Sequencing data for metataxonome and metatranscriptome

The kimchi samples were isolated to USM and NM by TFF for the metataxonomic diversity of USM and comparison with NM. The metataxonomic sequencing data for NM and USM were obtained (Table 1). The sequence profiles of the NM and USM...
TABLE 1  Conditions of sequence reads for each sample by metataxonomic and metatranscriptomic analyses.

| Analysis types         | Condition indices | USM     | NM      |
|------------------------|-------------------|---------|---------|
|                        | Raw sequence      | 47,555,685 | 2,094,071,926 |
|                        | Trimmed sequence read bases (bp) | 2,786 | 459 |
|                        | Total reads       | 197,783 | 891,252 |
|                        | Mean sequence read length (bp) | 1,279,587 | 42 |
|                        | GC contents (%)   | 43 | 36,346 |
|                        | Predicted protein features | 42 | 36,346 |
|                        | Predicted rRNA features | 805 | 2,782 |

USM, ultra-small microbiome; NM, normal microbiome.

were obtained as 61,700,786 and 52,968,776 bp, respectively. After merging and trimming, the total read bases and reads of NM were 854,180 bp and 1,840, and the mean sequence read length was 465 bp, while the GC content was 51%. The total read bases and total reads of USM were 1,279,587 bp and 2,786, and the mean sequence read length was 459 bp, while the GC content was 52%.

Metatranscriptomic sequencing data for NM and USM were obtained for identification of the potential functional transcriptome of USM and comparison with NM (Table 1). The sequence profiles of the NM and USM were obtained as 2,349,741,804 and 2,094,071,926 bp, respectively. After merging and trimming, the total read bases and reads of NM were 219,645,260 bp and 891,252, and the mean sequence read length was 246 bp. The GC content, predicted protein features, and predicted rRNA features were 42%, 147,923, and 2,782, respectively. The total read bases and total reads of USM were 47,555,685 bp and 197,783, and the mean sequence read length was 240 bp. The GC content, predicted protein features, and predicted rRNA features were 43%, 36,346, and 805, respectively.

Diversity of the ultra-small microbiome

The diversity of the USM in the kimchi sample was compared with NM (Figure 1; Supplementary Table 1) by metataxonomic analysis, and the pH in the kimchi sample was 4.13. The alpha diversity of each taxon is shown in Table 2. The observed operational taxonomic unit (OTU) for USM was higher than that for NM (168 vs. 118). Further, the Chao1, abundance-based coverage estimators (ACE), Shannon, Simpson, and Fisher indices of the USM were 304, 324, 1.64, 0.57, and 20.3, respectively, and those for NM were 188, 194, 0.88, 0.37, and 13.2, respectively.

The bacterial communities on the phylum level of the USM were dominated by Firmicutes (88.48%; Figure 1A), while Proteobacteria (3.79%), Bacteroidetes (1.42%), and Actinobacteria (1.28%) were not so dominant (Figure 1A). The bacterial communities of NM were dominated by Firmicutes (99.72%), just like the USM (Figure 1A). The bacterial communities at the genus level of the USM were dominated by Lactobacillus (64.52%), while Leuconostoc (12.99%), Weissella (5.63%), and Clostridium (2.21%) were not so dominant (Figure 1B). Lactobacillus (78.82%) was dominant in NM, while Weissella (14.07%) and Leuconostoc (6.30%) were not so dominant (Figure 1B).

At the species level, 41 species overlapped in the USM and NM, while 136 species were found only in USM, and 86 species were found only in NM (Figure 1C; Supplementary Table 2). Most of the overlapping species were those belonging to the genera Lactobacillus, Leuconostoc, and Weissella. Specifically, the relative ratio of Leuconostoc, which is known to have many strains that enhance the unique flavor of kimchi in the early stages of fermentation due to its good mannitol production ability (Lee et al., 2020), was significantly higher in the USM than in the NM. It is estimated that the pH of kimchi is 4.13 (Park et al., 2014), but since kimchi reached the ripe stage and Lactobacillus occupied the ecosystem, it is estimated that Leuconostoc was incorporated into the USM by reducing their size for survival.

The Chao1, ACE, Shannon, Simpson, and Fisher indices indicating alpha diversity were all higher in USM than in NM. These results were identical to those of a previous study (Lee et al., 2022), where the USMs of non-sterilized fermented cabbage varied. From these results, it was deduced that the diversity of the microbiome members below the 0.2 μm size defined by USM was not low. In both USM and NM, Lactobacillus, Leuconostoc, and Weissella belonging to lactic acid bacteria (LAB) were dominant, and this was presumed to be the ripening state with the most Lactobacillus spp. in LAB because of the pH of 4.1 in the kimchi sample known for having the high LAB content (Park et al., 2014). In addition, the genus Akkermansia, belonging to the phylum Verrucomicrobia, was detected only in the USM (Figure 1; Supplementary Table 1). Although Akkermansia was detected in a very small amount (0.05%), it was determined that there was a possibility that only USM could be purified and used as a new probiotic since it has just been described as a beneficial bacteria (Cani et al., 2022). However, since Akkermansia was not detected in the kimchi USM in the previous study (Lee et al., 2022), we believe that additional research is needed.
Contrary to the previous study (Lee et al., 2022), TM7, called Saccharibacteria, was not found in the kimchi sample of this study. It was probably detected in the previous study due to the single-molecule real-time method used, which could read all 16 rRNA genes. In this study, the clonal bridge amplification method that can read only relatively short sequences was used. TM7 may not have been found because only the V3 and V4 regions of the 16S rRNA gene were read by sequencing (Brown et al., 2016; Jeong et al., 2021). Also, some of the members of the USM might have been outer membrane vesicles (OMVs), which are 20 to 200 nm in size and are known to contain partial DNA and RNA (Schwechheimer and Kuehn, 2015; Reimer et al., 2021). Gram-negative bacteria known to produce OMVs (Jan, 2017) have not been detected in USM. However, there is still a possibility that some of the members of the USM identified in this study were OMVs, as there is a study that shows that gram-positive bacteria also emit OMVs (Schwechheimer and Kuehn, 2015).

**FIGURE 1**
Relative abundance profiling of microbiome analyzed in the USM and NM. Relative abundance profiling of microbial communities was analyzed at the phylum level (A) and genus level (B). A Venn diagram confirmed the relationship between samples on the species level (C).
TABLE 2 The alpha diversities of each sample identified by metataxonomic analysis.

| Indices  | USM | NM |
|---------|-----|----|
| Observed OTU | 168 | 118 |
| Chao1   | 304 | 188 |
| ACE     | 324 | 194 |
| Shannon | 1.64 | 0.88 |
| Simpson | 0.57 | 0.37 |
| Fisher  | 20.3 | 13.2 |

OTU, operational taxonomic unit; ACE, abundance-based coverage estimators; USM, ultra-small microbiome; NM, normal microbiome.

Analysis of the potential functional transcriptome

Comparison of transcriptomes of USM and NM

Metatranscriptome analysis has been used for the investigation of functional and taxonomic dynamics in fermented foods such as hard cheese (Quijada et al., 2022), camembert cheese (Lessard et al., 2014), liquor starter (Huang et al., 2017), pao cai (Xiao et al., 2021), and kimchi (Jung et al., 2013). The transcriptomes of USM in the kimchi sample were compared with those of the NM using metatranscriptomic analysis with the SEED subsystem (Figure 2A; Supplementary Table 3). Based on that subsystem in the USM, the metatranscriptome corresponding to level 1 was dominated by transcriptomes related to “protein metabolism” (27.70%), carbohydrates (19.11%), and “clustering-based subsystems” (10.93%; Figure 2A). The “cell wall and capsule” (4.52%), “cofactors, vitamins, prosthetic groups, pigments” (4.43%), “RNA metabolism” (3.92%), “respiration” (3.62%), “stress response” (3.34%), “virulence, disease and defense” (2.80%), “DNA metabolism” (2.71%), “membrane transport” (2.53%), “amino acids and derivatives” (2.32%), “nucleosides and nucleotides” (2.23%), and “phages, prophages, transposable elements, plasmids” (1.30%) transcriptomes were minorly dominant in the USM (Figure 2A). The transcriptomes of NM were also mainly dominated by “protein metabolism” (39.10%), “Gram-positive cell wall components” (34.97%), “Gram-negative cell wall components” (2.95%), and “cell wall of mycobacteria” (2.35%; Figure 2B). In the NM, it was also occupied by RNA transcripts associated with “capsular and extracellular polysaccharides” (42.37%), “Gram-positive cell wall components” (23.55%), “cell wall of mycobacteria” (2.06%), and “Gram-negative cell wall components” (1.82%; Figure 2B).

Transcriptomes related to “cell wall and capsule”

In the USM, the level 2 transcriptome associated with the “cell wall and capsule” was occupied by RNA transcripts associated with “capsular and extracellular polysaccharides” (39.10%), “Gram-positive cell wall components” (34.97%), “Gram-negative cell wall components” (2.95%), and “cell wall of mycobacteria” (2.35%; Figure 2B). In the NM, it was also occupied by RNA transcripts associated with “capsular and extracellular polysaccharides” (42.37%), “Gram-positive cell wall components” (23.55%), “cell wall of mycobacteria” (2.06%), and “Gram-negative cell wall components” (1.82%; Figure 2B).

Transcriptomes related to “stress response”

In the USM, the level 2 transcriptome associated with the “stress response” was occupied by transcripts associated with “oxidative stress” (44.7%), “osmotic stress” (22.9%), “heat shock” (13.0%), and “cold shock” (4.3%; Figure 2B). In the NM, it was occupied by transcripts associated with “osmotic stress” (34.1%), “oxidative stress” (28.1%), “heat shock” (22.0%), “cold shock” (2.4%), and “detoxification” (1.4%; Figure 2B).

Transcriptomes related to “virulence, disease, and defense”

In the USM, the level 2 transcriptome associated with “virulence, disease and defense” was occupied by transcripts associated with “resistance to antibiotics and toxic compounds” (57.9%), “invasion and intracellular resistance” (10.8%), “bacteriocins (ribosomally-synthesized antibacterial peptides)” (6.6%), and “adhesion” (5.4%; Figure 2B). In the NM, it was occupied by transcripts associated with “resistance to antibiotics and toxic compounds” (66.5%), “invasion and intracellular resistance” (13.0%), “adhesion” (4.8%), and “bacteriocins (ribosomally-synthesized antibacterial peptides)” (3.6%; Figure 2B).

Transcripts at the functional level

The transcripts at the functional level of the USM and NM overlapped (48.2%), and for the USM, only 3.2% had unique transcripts, while 37.9% of the NM had unique transcripts. However, the abundance of transcripts was not considered (Figure 2C; Supplementary Table 3).
Comparison of transcriptome related to survival

Since the formation of USM is thought to be related to survival, the lower transcripts were identified in the functional classification categories of level 1 “cell wall and capsule,” “stress response,” and “virulence, disease, and defense” and compared with the NM. In the “cell wall and capsule,” the transcripts related to “Gram-negative cell wall components” and “Gram-positive cell wall components” were analyzed. The relative abundance profiling of metatranscriptome was analyzed in the USM and NM, showing differences in the transcript composition between the two samples.

**FIGURE 2**
Relative abundance profiling of metatranscriptome analyzed in the USM and NM. The relative abundance profiling of transcripts was analyzed in level 1 (A), and the relative abundance profiling of sub transcripts related to “cell wall and capsule,” “stress response,” and “virulence, disease, and defense” was analyzed in level 2 (B). A Venn diagram confirmed the relationship between samples on the functional level (C).
wall components" had a relatively higher proportion in the USM than in the NM. It was possible that as the cell size became small enough to belong to the USM, the ratio of the transcripts related to the cell wall components became relatively high. The transcripts related to "oxidative stress" in "stress response" had a relatively higher proportion in the USM than in the NM. Some studies (Desnues et al., 2003; Yoon et al., 2020) have shown that many oxidative stress-related proteins are expressed since VBNC is formed due to oxidative stress. Therefore, it was deduced that there were more bacteria in the VBNC state of the USM than that of the NM. In contrast, the relative proportion of transcripts related to "virulence, disease, and defense" category was lower in the USM (2.80%) than in the NM (4.05%). The ratio of transcripts related to "resistance to antibiotics and toxic compounds" in "virulence, disease, and defense" was also lower in the USM than in the NM. In general, microorganisms in the VBNC state cannot attach to enterocytes or plastics and cannot form biofilms (Pruzzo et al., 2003; Lleo et al., 2007) as they may lose their infectivity or become toxic (Oliver and Bockian, 1995; Lindbäck et al., 2010). From these previous studies, if a member contains a lot of bacteria in the VBNC state, the expression of transcripts related to "virulence, disease, and defense" becomes low. Therefore, it was presumed that there are many bacteria in the VBNC state in the members of the USM. Furthermore, some bacteria in the USM may be OMVs because the size of OMVs with luminal DNA is small (100–300 nm; Jan, 2017). However, transcriptome transcribed into mRNA was thought to be evidence that USM is alive and exists (Li et al., 2014). Since the types of transcripts identified in the USM at the functional level were less than those in NM (Figure 2C), they were thought to minimize the types of proteins expressed in the USM.

**RNA virome**

In addition, the RNA virome of USM in the kimchi sample was compared with NM by metatranscriptomic analysis using RefSeq (Figure 3; Supplementary Table 4) by the Virus Metadata Resource (VMR) based on the International Committee on Taxonomy of Viruses (ICTV). The RNA viral communities on the genus level of the USM were mainly dominated by Tobamovirus (82.09%) and Allexivirus (9.35%), Carlavirus (2.63%) and Nepovirus (1.93%) were minorly dominant in the USM (Figure 3A). The RNA viral communities on the genus level of the NM were mainly dominated by Carlavirus (33.98%), Allexivirus (26.01%), and Tobamovirus (25.63%). Those with minor dominance in the NM were Tospovirus (2.99%), Fabavirus (2.84%), Fovavirus (2.58%), and Potyvirus (1.77%; Figure 3A). Further, at the species level, pepper mild mottle virus (PMMoV) was dominant in both the USM (80.10%) and NM (25.45%; Figure 3B).

**Correlation between metataxonome and metatranscriptome identified USM and NM**

The correlation analysis between metataxonome and metatranscriptome was expressed as a heatmap (Figure 4; Supplementary Table 5). Transcripts related to "virulence, disease, and defense" was negatively correlated with Bacteroides (r = −0.51) at the genus level. Transcripts related to "stress response" were negatively correlated with Butyricimonas (r = −0.52) and Delftia (r = −0.57). Both positive and negative correlation coefficients did not exceed 0.5 large in transcripts related to "cell wall and capsule," so it was considered that the correlation with bacteria at the genus level was not.

Transcripts of categories of "virulence, disease and defense" and "stress response," which are judged to be related to survival, were negatively correlated with bacteria. Genus Bacteroides negatively correlated (r = −0.51) with "virulence, disease and defense" was found only in USM (Figure 1B; Supplementary Table 1), and it can be seen that "virulence, disease, and defense" also expressed relatively low USM compared to NM (Figure 2A). Genera Butyricimonas and Delftia negatively correlated (r = −0.52 and r = −0.57) with "stress response" were found only in USM (Figure 1B; Supplementary Table 1), respectively. It can also be seen that "stress response" expressed relatively low USM compared to NM (Figure 2A). Bacteria in the metataxonome that were correlated with "cell wall and capsule" did not appear.

Transcripts related to "sulfur metabolism" were negatively correlated with Acidobacterium (r = −0.51) and Hespellia (r = −0.57). Transcripts related to "secondary metabolism" were positively correlated with Lactococcus (r = 0.52) and Terrimonas (r = 0.55), were negatively correlated with Halomonas (r = −0.53) and Staphylococcus (r = −0.51). Transcripts related to "respiration" were positively correlated with Leuconostoc.

Viruses such as Tobamovirus, Carlavirus, and Allexivirus, which are mainly parasitic on plants (Hulo et al., 2011), accounted for the majority. Since the samples in this study were mostly kimchi made of plant materials, the USM and NM were thought to have a high ratio of plant viruses (RNA viruses). However, PMMoV belonging to Tobamovirus was highly dominant in the USM and NM. In a study by Kim et al. (2014), it was found that most of the plant RNA viruses of kimchi were PMMoV, which is consistent with our results. Moreover, the reason for the high ratio of PMMoV as the major pathogenic virus of plants in the genus Capsicum (Jarret et al., 2008) in the USM and NM was presumed to be due to the chili powder used in kimchi. Although the RNA virome was identified through metatranscriptome analysis in this study, it could not be related to the metataxonome. In addition, although prophage forms exist in bacterial cells, free RNA viruses are also presumed to exist, so it was judged that the difference between the USM and the NM might be a coincidence.

**References**

1. Desnues et al., 2003
2. Lleo et al., 2007
3. Jarret et al., 2008
4. Kim et al. (2014)
5. https://ictv.global/
Transcripts related to “regulation and cell signaling” were positively correlated with *Cobetia* (*r* = 0.54), *Fusobacterium* (*r* = 0.58) and *Legionella* (*r* = 0.59), were negatively correlated with *Butyrivibrio* (*r* = −0.52). Transcripts related to “protein metabolism” were negatively correlated with *Bacillus* (*r* = −0.52). Transcripts related to “potassium metabolism” were positively correlated with *Lactobacillus* (*r* = 0.54), and were negatively correlated with *Megamonas* (*r* = −0.57). Transcripts related to “photosynthesis” were positively correlated with *Enterobacter* (*r* = 0.67), *Pectobacterium* (*r* = 0.57) and *Weissella* (*r* = 0.57). Transcripts related to “phosphorus metabolism” were positively correlated with *Abiotrophia* (*r* = 0.65) and *Coenonia* (*r* = 0.57), were negatively correlated with *Salinivibrio* (*r* = −0.57). Transcripts related to “phages, prophages, transposable elements, plasmids” were positively correlated with *Bacillus* (*r* = 0.57) and *Faecalibacterium* (*r* = 0.52), were negatively correlated with *Pedobacter* (*r* = −0.59). Transcripts related to “nucleosides and nucleotides” were negatively correlated with *Ruminococcus* (*r* = −0.66). Transcripts related to “nitrogen metabolism” were negatively correlated with *Cronobacter* (*r* = −0.54) and *Weissella* (*r* = −0.51). Transcripts related to “motility and chemotaxis” were positively correlated with *Eubacterium* (*r* = 0.52), and were negatively correlated with *Capnocytophaga* (*r* = −0.55). Transcripts related to “metabolism
of aromatic compounds” were positively correlated with Sarcina (r = 0.51). Transcripts related to “Membrane Transport” were positively correlated with Ethanoligenens (r = 0.60), and were negatively correlated with Terrimonas (r = −0.57). Transcripts related to “iron acquisition and metabolism” were positively correlated with Catenibacterium (r = 0.50) and Chryseobacterium (r = 0.54), and were negatively correlated with Agrobacterium (r = −0.55). Transcripts related to “fatty acids, lipids, and isoprenoids” were positively correlated with Acidobacterium (r = 0.53), and were negatively correlated with Planktothrix (r = −0.51), Staphylococcus (r = −0.55) and Stenotrophomonas (r = −0.64). Transcripts related to “dormancy and sporulation” were negatively correlated with Bradyrhizobium (r = −0.57), Sarcina (r = −0.56) and Streptomyces (r = −0.56). Transcripts related to “DNA metabolism” were positively correlated with Dyella (r = 0.53), and were negatively correlated with Catenibacterium (r = −0.57) and Legionella (r = −0.60). Transcripts related to “cell division and cell cycle” were negatively correlated with Stenotrophomonas (r = −0.60). Transcripts related to “amino acids and derivatives” were negatively correlated with Robinsoniella (r = −0.58). In addition, both positive and negative correlation coefficients did not exceed 0.5 in transcripts related to “RNA metabolism,” “cofactors, vitamins, prosthetic groups, pigments” and “carbohydrates,” so it was considered that the correlation with bacteria at genus level was not.

Since “sulfur metabolism” and “dormancy and sporulation” only showed a negative correlation with bacteria (genera Acidobacterium and Hespellia for “sulfur metabolism”; genera Bradyrhizobium, Sarcina, and Streptomyces for “dormancy and sporulation”) belonging to USM, there is a possibility that the expression level of A-related transcripts is low in USM. On the other hand, “membrane transport” showed a positive correlation only with bacteria (genera Ethanoligenens and Terrimonas) belonging to USM, and it is possible that the expression level of transcripts related to “membrane transport” in USM is relatively higher than that of NM. Also, there was no relationship between metataxonome and metatranscriptome with positive or negative correlation coefficients greater than 0.8, but there were relationships with positive or negative correlation coefficients greater than 0.5. Therefore, it is thought that the metataxonome and metatranscriptome influence each other to some extent. However, only limited samples were obtained due to the difficulty of pretreatment. To overcome this, since the correlation was analyzed with samples obtained by bootstrapping, more research is needed to obtain clearer results.

Conclusion

In this study, we compared the USM metataxonome and metatranscriptome of kimchi, passed through a filter with pores of 0.2 μm or less using TFF, to those of NM. From our results, the USM and NM of kimchi showed different microbial diversity, and transcripts were also expressed differently. Further, unlike the NM, the metatranscriptome of the USM showed some of the characteristics of VBNC
confirmed in previous studies, so it was estimated that there were many bacteria in the VBNC state of the USM. While the results of the metatranscriptomic and metataxonomic analyses of the USM can be controversial, it should always be considered that a small number of microorganisms trying to survive in a colony of other microbes have also formed their separate colonies too. Based on these analyses, viruses and transcriptomes of the UMB, OMV, and bacteria of VBNC states, which are expected to be mixed in the USM, were studied. This is considered a stepping stone that can contribute greatly to related research in the future.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, SRR21133393; https://www.ncbi.nlm.nih.gov/, SRR21133394; https://www.ncbi.nlm.nih.gov/, SRR21133488; and https://www.ncbi.nlm.nih.gov/, SRR21133489.

Author contributions

H-WL: writing—original draft, investigation, data curation, conceptualization, software, methodology, and funding acquisition. S-RY: writing—review and editing, and investigation. Y-MD: investigation and data curation. J-HY: investigation, methodology, and software. HJ and K-NK: methodology and data curation. J-HH: validation, resources, writing—review and editing, funding acquisition, and supervision. J-WB: writing—review and editing, data curation, and supervision. All authors contributed to the article and approved the submitted version.

References

Ayrapetyan, M., Williams, T., and Oliver, J. D. (2018). Relationship between the viable but nonculturable state and antibiotic persister cells. J. Bacteriol. 200, e00249–e00218. doi: 10.1128/JB.00249-18

Babin, B. M., Åtackh, L., van Eldik, M. B., Swederoski, M. J., Moradian, A., Heo, S., et al. (2017). Selective proteomic analysis of antibiotic-tolerant cellular subpopulations in Pseudomonas aeruginosa biofilms. mBio 8:e01593-17. doi: 10.1128/mBio01593-17

Bikel, S., Valdez-Lara, A., Cornejo-Granados, F., Rico, K., Canizales-Quinteros, S., Soberón, X., et al. (2015). Combining metagenomics, metatranscriptomics and viromics to explore novel microbial interactions: towards a systems-level understanding of human microbiome. Comput. Struct. Biotechnol. J. 13, 390–401. doi: 10.1016/j.csbj.2015.06.001

Bollen, C., Dewachter, L., and Michiels, J. (2019). Protein aggregation as a bacterial strategy to survive antibiotic treatment. Front. Mol. Biosci. 8:66964. doi: 10.3389/fmolb.2021.66964

Bousquet, J., Anto, J. M., Carlewska, W., Haahida, T., Fonseca, S. C., Jaccarino, G., et al. (2021). Cabbage and fermented vegetables: from death rate heterogeneity in countries to candidates for mitigation strategies of severe COVID-19. Allergy 76, 735–750. doi: 10.1111/all.15459

Brown, C. T., Olm, M. R., Thomas, B. C., and Banfield, J. F. (2016). Measurement of bacterial replication rates in microbial communities. Nat. Biotechnol. 34, 1256–1263. doi: 10.1038/nbt.3704

Cai, L., Yang, Y., Jiao, N., and Zhang, R. (2015). Evaluation of tangential flow filtration for the concentration and separation of bacteria and viruses in contrasting marine environments. PLoS One 10:e0136741. doi: 10.1371/journal.pone.0136741

Cani, P. D., Depommier, C., Derrien, M., Everard, A., and de Vos, W. M. (2022). Akkermansia muciniphila: paradigm for next-generation beneficial microorganisms. Nat. Rev. Gastroenterol. Hepatol. 19, 625–647. doi: 10.1038/s41575-022-00631-9

Das, G., Heredia, J. B., de Lourdes Pereira, M., Coy-Barrera, E., Rodrigues Oliveira, S. M. R., Gutiérrez-Grijalva, E. F., et al. (2021). Korean traditional foods as antiviral and respiratory disease prevention and treatments: A detailed review. Trends Food Sci. Technol. 116, 415–433. doi: 10.1016/j.tifs.2021.07.037

De Filippis, F., Valentino, V., Alvarez-Ordóñez, A., Cotter, P. D., and Ercolini, D. (2021). Environmental microbiome mapping as a strategy to improve quality and safety in the food industry. Curr. Opin. Food Sci. 38, 168–176. doi: 10.1016/j.cofo.2020.11.012

Desnues, B., Cuny, C., Grégori, G., Dukan, S., Aguilaniu, H., and Nyström, T. (2003). Differential oxidative damage and expression of stress defence regulons in culturable and non-culturable Escherichia coli cells. EMBO Rep. 4, 400–404. doi: 10.1038/sj.embor.7400799

Dharirwal, A., Chong, J., Habib, S., King, I. L., Agellon, L. B., and Xia, J. (2017). Microbiome analyst: a web-based tool for comprehensive statistical, visual and

Funding

This work was supported by the National Research Foundation of Korea (NRF-2019R1F1A1061368) and the World Institute of Kimchi (KE2202-2).

Acknowledgments

We thank Macrogen (South Korea) for their assistance with the metatranscriptomic and metataxonomic sequencing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher’s note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1026513/full#supplementary-material
meta-analysis of microbiome data. *Nucleic Acids Res.* **45**, W180–W188. doi: 10.1093/nar/gkx295

Du, M., Chen, J., Zhang, X., Li, A., Li, Y., and Wang, Y. (2007). Retention of virulence in a viable but nonculturable *Edwardsiella tarda* isolate. *Appl. Environ. Microbiol.* **73**, 1349–1354. doi: 10.1128/AEM.02243-06

Gallardo-Becerra, L., Cornejo-Granados, F., García-López, R., Valdez-Lara, A., Bikel, S., Canizales-Quinteros, S., et al. (2020). Metatranscriptomic analysis to define the Scredrome, and 165 rRNA profiling of the gut microbiome in obesity and metabolic syndrome of Mexican children. *Microbiol. Cell Fact.* **19**:61. doi: 10.1186/s12934-020-01319-y

Ghuneim, L. I., Jones, D. L., Golyshin, P. N., and Golyshina, O. V. (2018). Nano-sized and filterable bacteria and archaea: biodiversity and function. *Front. Microbiol.* **9**:1971. doi: 10.3389/fmicb.2018.01971

Huang, Y., Yi, Z., Jin, Y., Huang, M., He, K., Liu, D., et al. (2017). Metatranscriptomics reveals the functions and enzyme profiles of the microbial community in Chinese nong-flavor liquor starter. *Front. Microbiol.* **8**:1747. doi: 10.3389/fmicb.2017.01747

Hulo, C., de Castro, E., Masson, P., Bouguerel, L., Barroch, A., Xanarios, J., et al. (2011). Viral zone: a knowledge resource to understand virus diversity. *Nucleic Acids Res.* **39**. doi: 10.1093/nar/gkq842

Jan, A. T. (2017). Outer membrane vesicles (OMV) of gram-negative bacteria: A perspective update. *Front. Microbiol.* **8**:1053. doi: 10.3389/fmicb.2017.01053

Jarret, R., Gillaspie, A., Barkley, N., and Pinnov, D. (2008). The occurrence and control of pepper mild mottle virus (PMMoV) in the USDA/ARS Capsicum germplasm collection. *Seed Technol.* **30**, 26–36.

Jeong, J., Yun, K., Mun, S., Chung, W. H., Choi, S. Y., Nam, Y. D., et al. (2021). The effect of taxonomic classification by full-length 16S rRNA sequencing with a synthetic long-read technology. *Sci. Rep.* **11**:17127. doi: 10.1038/s41598-020-08082-9

Jung, J. Y., Lee, S. H., Jin, H. M., Hahn, Y., Madsen, E. L., and Jeon, C. O. (2013). Metatranscriptomic analysis of lactic acid bacterial gene expression during kimchi fermentation. *Int. J. Food Microbiol.* **163**, 171–179. doi: 10.1016/j.ijfoodmicro.2013.02.022

Kim, E. K., Ha, A. W., Choi, E. O., and Jo, S. Y. (2016). Analysis of kimchi, vegetable and fruit consumption trends among Korean adults: data from the Korea National Health and Nutrition examination survey (1998–2012). *Nutr. Res. Pract.* **10**, 188–197. doi: 10.4162/nrup.2016.10.188

Kim, D. S., Jung, J. Y., Wang, Y., Oh, H. J., Choi, D., Jeon, C. O., et al. (2014). Plant virus RNA sequences identified in kimchi by microbial metatranscriptome analysis. *I. Microbiol. Biotechnol.* **24**, 979–986. doi: 10.4103/1444-0401.140017

Kwon, D. Y., Jung, D. J., Yang, H. J., and Chung, K. R. (2014). History of Korean gochu, gochujang, and kimchi. *J. Ethn. Foods.* **24**, 979–986. doi: 10.4103/1444-0401.140017

Lee, K. W., Kim, G. S., Baek, A. H., Hwang, H. S., Kwon, D. Y., Kim, S., et al. (2020). Isolation and characterization of kimchi starters *Leuconostoc mesenteroides* PBio03 and *Leuconostoc mesenteroides* PBio04 for manufacture of commercial kimchi. *J. Microbiol. Biotechnol.* **30**, 1066–1066. doi: 10.4014/jmb.2001.01011

Lee, C. H., and Ko, Y. J. (2021). A new process on the basic formula of kimchi: derived kimchi from a combination of yam yangnyeo (kimchi sauce) and vegetables. *I. Ethn. Foods.* **8**, 1–12. doi: 10.4162/ijef.2021.001010

Lee, H.-W., Yoon, S.-R., Dang, Y.-M., Kang, M., Kang, K. H., Ha, J., et al. (2022). Analysis of kimchi, shrimp, seaweed, and rice metabolism transcriptional profiles in artisanal Austrian hard-cheeses during ripening. *Front. Microbiol.* **13**:813. doi: 10.3389/fmicb.2022.813480

Nakai, R. (2020). Size matters: ultra-small and filterable microorganisms in the environment. *Microbiomes*. doi: 10.25552/microbio1352

Pang, Z., Chong, J., Zhou, G., de Lima Morais, D. A., Chang, L., Barrette, M., et al. (2020). Metatranscriptome analysis to define virulence in a viable but nonculturable *Vibrio parahaemolyticus*. *Front. Microbiol.* **11**, 3620–3623. doi: 10.3389/fmicb.2020.103573

Pluizel, F., Paarmann, D., D’Souza, M., Olson, R., Glass, E. M., Kubal, M., et al. (2008). The metagenomics RAST server - a public resource for the comprehensive phylogenetic and functional analysis of metagenomes. * BMC Bioinformatics.* **9**:386. doi: 10.1186/1471-2105-9-386

Saeed, A. I., Sharov, V., White, J., Li, J., Liang, W., Bhagabati, N., et al. (2003). TM4: A free, open-source system for microarray data management and analysis. *Biotecniques* **34**, 374–378. doi: 10.2144/0342m019