

Plant RNA Virus Sequences Identified in *Kimchi* by Microbial Metatranscriptome Analysis^S

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Plant pathogenic RNA viruses are present in a variety of plant-based foods. When ingested by humans, these viruses can survive the passage through the digestive tract, and are frequently detected in human feces. *Kimchi* is a traditional fermented Korean food made from cabbage or vegetables, with a variety of other plant-based ingredients, including ground red pepper and garlic paste. We analyzed microbial metatranscriptome data from *kimchi* at five fermentation stages to identify plant RNA virus-derived sequences. We successfully identified a substantial amount of plant RNA virus sequences, especially during the early stages of fermentation: 23.47% and 16.45% of total clean reads on days 7 and 13, respectively. The most abundant plant RNA virus sequences were from pepper mild mottle virus, a major pathogen of red peppers; this constituted 95% of the total RNA virus sequences identified throughout the fermentation period. We observed distinct sequencing read-depth distributions for plant RNA virus genomes, possibly implying intrinsic and/or technical biases during the metatranscriptome generation procedure. We also identified RNA virus sequences in publicly available microbial metatranscriptome data sets. We propose that metatranscriptome data may serve as a valuable resource for RNA virus detection, and a systematic screening of the ingredients may help prevent the use of virus-infected low-quality materials for food production.

Keywords: *Kimchi*, plant RNA virus, metatranscriptome, pepper mild mottle virus

Introduction

Plant pathogenic RNA viruses are present in a variety of plant-based foods and consumables, such as fresh produce, fermented vegetables, and tobacco products [2, 13, 23, 30]. Plant RNA viruses cause drastic reductions in fruit, crop, and vegetable production [3, 7, 11, 21]. The estimated economic loss in crop yield due to plant RNA viruses is about 60 billion US dollars worldwide each year [28]. Pepper mild mottle virus (PMMoV), for example, was widely detected in pepper plants, fruits, and seeds [3, 14, 27, 29]. Symptoms caused by PMMoV include mottling, curling, dwarfing, and distortion of pepper fruits and leaves [4, 25, 29].

Plant RNA viral genomic nucleic acids are encapsulated in highly stable multifunctional protein coats [1]. When

ingested by humans, plant RNA viruses can survive the passage through the digestive tract and are frequently detected in human feces [6, 9, 31]. For example, PMMoV particles and genomic sequences were reported to be present in high numbers in human fecal samples [4]. PMMoVs were also detected globally in environmental and drinking water sources, including those in Korea, and their presence along with other viruses has been proposed as an indicator of human fecal contamination [9, 10, 12, 24].

Plant RNA viruses are not generally considered to be pathogenic and have been used to produce vaccines for humans and animals [5, 8]. However, there is growing evidence that plant viruses may be associated with clinical symptoms of diseases in humans [4, 17, 19]. For example, it was reported that PMMoV might be associated with specific immune responses, such as fever, abdominal pains,

and pruritus [4]. Another plant RNA virus, tobacco mosaic virus (TMV), was also reported to affect human health, and individuals who smoked showed high levels of antibodies against TMV proteins [17]. It has also been proposed that high levels of TMV antibodies might be associated with a lower risk of developing Parkinson's disease [17, 18, 20]. These examples suggest that some plant RNA viruses may affect human health.

Kimchi is a traditional Korean fermented food made of vegetables such as cabbage and radish, with a variety of plant-based ingredients, including ground red pepper, garlic paste, minced ginger, and chopped green onion, added to it. Since *kimchi* is made from a large variety of plant matter, depending on the quality of the source materials, *kimchi* may contain plant RNA viruses. To test this possibility, we analyzed microbial metatranscriptome data obtained from *kimchi* during five different stages of its fermentation [15] and identified sequences derived from plant RNA viruses.

Materials and Methods

Preparation of Reference RNA Virus Sequences

Complete genome sequences were downloaded from the virus division of the National Center for Biotechnology Information (NCBI) RefSeq database. The search term for the NCBI Nucleotide database was "complete [Title] AND gbdiv vrl [Properties] AND srdb refseq [Properties]." Sequences isolated from RNA viruses were extracted by checking the "LOCUS" line of the NCBI GenBank-format records. The resulting complete RNA virus genome sequences were converted to a BLAST-searchable database.

Microbial Metatranscriptome Data

Kimchi microbial metatranscriptome data from five fermentation stages (on days 7, 13, 18, 25, and 29) were previously prepared and analyzed in detail [15]. Sample names were J7, J13, J18, J25, and J29, respectively, where the number indicates the days after *kimchi* stock preparation. The five metatranscriptome sequence data sets are available at the NCBI Short Read Archive (SRA), under accession numbers SRX128699, SRX128700, SRX128702, SRX128704, and SRX128705, respectively. As described in the previous report [15], raw read sequences were processed to obtain "clean" reads. Briefly, the first base of each read was removed because an ambiguous base "N" appeared at the start of most of the reads. Then, the reads were trimmed to remove bases with a quality score of <20 using the FASTX-Toolkit software (http://hannonlab.cshl.edu/fastx_toolkit). Finally, short reads (<50 bp) were discarded. The number of resulting clean reads ranged from 30.5–35.3 million for each of the five samples. These clean reads were assigned to either a structural RNA (rRNAs or tRNAs) or a gene from six highly dominant lactic acid bacteria (LAB), including

Leuconostoc (Lc.) mesenteroides, *Lactobacillus sakei*, *Weissella koreensis*, *Lc. gelidum*, *Lc. carnosum*, and *Lc. gasicomitatum* [15]. The unassigned reads were collected and used as the input data for identification of plant RNA viruses.

Fifty-nine sequencing runs of microbial metatranscriptome data isolated from human fecal samples (SRA Accession No. SRA075676), reported to contain high numbers of tomato mosaic virus (ToMV) sequences, were downloaded [6]. These sequences were analyzed to obtain ToMV sequencing read-depth distributions. All the reads in this data set were paired-end. Because *kimchi* microbial metatranscriptome sequences were single-end, only one end of each read was used.

To identify RNA virus sequences, three additional microbial metatranscriptome data sets were obtained. These data were derived from cow rumen (six runs, SRA059441) [22], human gut (four runs, SRA072485) [16], and human fecal samples (six runs, SRX015725 and SRX015726) [26]. Cow rumen data were paired-end reads, but only one end was used; the other data were single-end.

Identification of RNA Virus Sequences in Microbial Metatranscriptome

BLASTN searches were performed to identify RNA virus sequences in the microbial metatranscriptome data derived from *kimchi*, cow rumen, human gut, and human fecal samples. BLASTN parameters were "-outfmt 10 -max_target_seqs 1 -evalue 1e-3 -perc_identity 90." Reads that aligned a viral genome sequence with 90% sequence identity or greater, and with query coverage of 90% or longer, were selected as matches. Matched reads were grouped based on the source virus; the "SOURCE" field of the NCBI GenBank-format record was used as the key.

Construction of the *Kimchi* PMMoV Consensus Sequence

Kimchi microbial metatranscriptome reads that matched the reference PMMoV genome sequence NC_003630 were collected. Reads with gaps or ambiguous nucleotides were discarded. For each nucleotide position, the number of reads for each of the four nucleotides was calculated. At each position, sequences exhibiting at least 10% of the total reads were retained. The positions where the *kimchi* PMMoV sequence was different from the reference genome were analyzed.

Results and Discussion

Plant RNA Virus Sequences in *Kimchi* Microbial Metatranscriptome Data

Previously, we reported gene-expression profiles of six dominant lactic acid bacteria (LAB) during *kimchi* fermentation by analyzing their metatranscriptome data [15]. Most of the metatranscriptome reads were mapped to the six LAB. However, a substantial number of reads was still not assigned as bacterial sequences. For a pilot analysis, we

randomly selected 100 non-bacterial sequences from the sample J7 and identified them using BLASTN searches of the NCBI nucleotide database “nr.” As a result, we found that 46% and 39% were derived from plants and plant RNA viruses, respectively. A further 9% were bacterial and 6% were unknown. We assumed that these plant and plant RNA virus sequences were concomitantly isolated during *kimchi* microbial RNA preparation. The pilot analysis result prompted us to systematically identify plant RNA virus sequences in *kimchi* microbial metatranscriptome data.

For the systematic identification of plant RNA virus sequences in *kimchi* microbial metatranscriptome data, BLASTN searches of the reference RNA virus genome sequence database were performed using all the unassigned metatranscriptome sequences as queries. The result showed that a significant portion of the metatranscriptome reads were derived from plant RNA viruses, especially during the early stages of fermentation (Table 1). RNA virus content reached 23.47% of the total clean reads on day 7 (sample J7); this was maintained at 16.45% on day 13 (sample J13). As the fermentation continued, RNA virus content declined to 2.36%, 3.75%, and 1.11% of the total clean reads on days 18, 25, and 29, respectively. The gradual decrease in the number of RNA virus sequences might be due to the overwhelming growth of fermenting bacteria that resulted in a gradual increase of the bacterial RNAs in the metatranscriptome data.

Almost all the RNA virus sequences in the *kimchi* metatranscriptome data were derived from plant RNA viruses, and only a small number of reads were from non-plant viruses; for example *Saccharomyces* viruses (Table 2 and Table S1). The most abundant plant RNA virus sequences identified was from PMMoV, which is a major pathogen of red peppers. This constituted 95% of total RNA virus sequences throughout the fermentation period. The second most abundant virus sequence was from garlic virus A (GarV-A), with a proportion of 2%. Small amounts of garlic common latent virus, broad bean wilt virus 2,

cucumber mosaic virus, garlic virus E, pepper mottle virus, and turnip mosaic virus sequences were also detected. Because the majority of virus sequences (>97%) were from PMMoV and GarV-A, we assumed that these virus sequences were derived from red peppers and garlic, respectively, which are both major and essential ingredients of *kimchi*. The overwhelming abundance of PMMoV and GarV-A might be explained by the use of pepper and garlic as a powder or paste, which might allow viral particles to be spontaneously released into the liquid fraction. Another possibility is that low-quality powdered peppers and minced garlic heavily infected with viruses were not removed from the ingredient material, either by chance or through negligence.

Sequencing Read-Depth Distributions

To examine whether *kimchi* PMMoV reads covered the entire genome, the depths of sequencing read at each nucleotide position were plotted (Fig. 1A). The read-depth plots revealed that PMMoV reads covered the entire region of the genome. However, the depth of the reads varied from position to position. Interestingly, the read-depth distributions of PMMoV from the five samples showed a strikingly similar pattern, although they were obtained from different *kimchi* batches and had different read numbers. The similarity of the read-depth patterns indicated that some regions of the viral genome were easier to sequence than the others, possibly due to the intrinsic RNA structure and stability, and/or biased RNA isolation, cDNA preparation, and/or sequencing reaction. The sequencing read-depth distributions of the *kimchi* GarV-A genome also showed a similar pattern among the five samples (Fig. 1B), confirming biased fragment isolation along the RNA viral genome.

To examine whether a similar sequencing read-depth distribution among different samples could be found from unrelated experiments, microbial metatranscriptome data obtained from human fecal samples reported to contain a

Table 1. Summary of *kimchi* microbial metatranscriptome data.

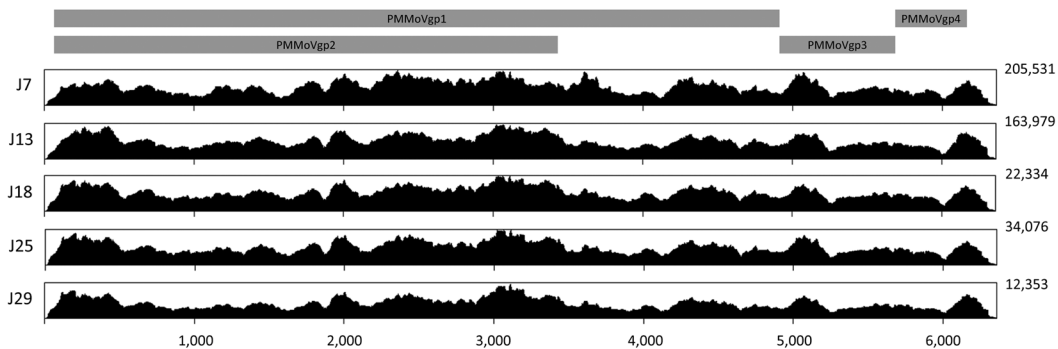
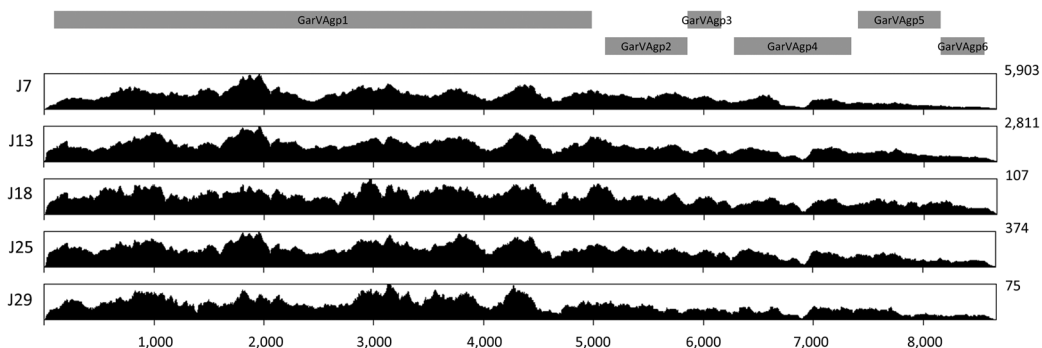
Sample	J7	J13	J18	J25	J29	Total
Clean reads	30,535,784	35,269,344	33,894,898	31,437,973	32,562,239	163,700,238
Structural RNA reads	10,337,105	18,618,452	23,074,547	27,187,841	26,768,731	105,986,676
%	33.85	52.79	68.08	86.48	82.21	64.74
Six LAB genome reads	425,552	5,332,645	8,442,826	1,768,222	3,869,480	19,838,725
%	1.39	15.12	24.91	5.62	11.88	12.12
RNA virus reads	7,166,178	5,801,188	799,652	1,179,043	361,863	15,307,924
%	23.47	16.45	2.36	3.75	1.11	9.35

Table 2. Distribution of *kimchi* microbial metatranscriptome reads derived from RNA viruses.

Virus	J7	%	J13	%	J18	%	J25	%	J29	%	Total	%
Pepper mild mottle virus	6,801,491	94.91	5,546,194	95.60	776,124	97.06	1,122,330	95.19	344,935	95.32	14,591,074	95.32
Garlic virus A	180,640	2.52	108,542	1.87	4,651	0.58	15,861	1.35	2,678	0.74	312,372	2.04
Garlic common latent virus	28,867	0.40	57,528	0.99	2,854	0.36	19,144	1.62	2,733	0.76	111,126	0.73
Broad bean wilt virus 2	46,813	0.65	24,793	0.43	5,401	0.68	6,421	0.54	4,130	1.14	87,558	0.57
Cucumber mosaic virus	51,215	0.71	15,403	0.27	6,089	0.76	4,797	0.41	4,535	1.25	82,039	0.54
Garlic virus E	18,058	0.25	17,373	0.30	925	0.12	2,752	0.23	487	0.13	39,595	0.26
Pepper mottle virus	9,303	0.13	4,755	0.08	791	0.10	1,072	0.09	444	0.12	16,365	0.11
Turnip mosaic virus	5,647	0.08	6,986	0.12	371	0.05	1,260	0.11	313	0.09	14,577	0.10
Beet western yellows virus	4,972	0.07	4,202	0.07	647	0.08	1,358	0.12	425	0.12	11,604	0.08
Garlic virus X	4,028	0.06	4,310	0.07	305	0.04	1,010	0.09	196	0.05	9,849	0.06
Garlic virus D	3,283	0.05	3,438	0.06	254	0.03	664	0.06	106	0.03	7,745	0.05
Paprika mild mottle virus	3,732	0.05	1,560	0.03	303	0.04	504	0.04	157	0.04	6,256	0.04
Tobacco mild green mosaic virus	1,970	0.03	1,257	0.02	122	0.02	515	0.04	120	0.03	3,984	0.03
Garlic virus C	1,821	0.03	1,640	0.03	98	0.01	335	0.03	53	0.01	3,947	0.03
Cucumber mosaic virus satellite RNA	964	0.01	74	0.00	293	0.04	82	0.01	272	0.08	1,685	0.01
Garlic latent virus	563	0.01	480	0.01	44	0.01	116	0.01	34	0.01	1,237	0.01
Tobacco mosaic virus	285	0.00	588	0.01	39	0.00	235	0.02	12	0.00	1,159	0.01
Turnip yellows virus	366	0.01	310	0.01	53	0.01	100	0.01	26	0.01	855	0.01
Bell pepper endornavirus	406	0.01	198	0.00	43	0.01	67	0.01	28	0.01	742	0.00
Leek yellow stripe virus	290	0.00	269	0.00	34	0.00	85	0.01	38	0.01	716	0.00
Beet mild yellowing virus	265	0.00	261	0.00	48	0.01	84	0.01	20	0.01	678	0.00
Brassica yellows virus	265	0.00	252	0.00	44	0.01	72	0.01	20	0.01	653	0.00
Gayfeather mild mottle virus	217	0.00	49	0.00	44	0.01	25	0.00	47	0.01	382	0.00
Beet chlorosis virus	140	0.00	133	0.00	17	0.00	42	0.00	19	0.01	351	0.00
Onion yellow dwarf virus	83	0.00	147	0.00	10	0.00	27	0.00	4	0.00	271	0.00
Tomato mosaic virus	42	0.00	143	0.00	9	0.00	20	0.00	2	0.00	216	0.00
Chilli vein mottle virus	120	0.00	62	0.00	9	0.00	12	0.00	9	0.00	212	0.00
Potato virus Y	155	0.00	35	0.00	2	0.00	15	0.00	4	0.00	211	0.00
Youcai mosaic virus	60	0.00	107	0.00	9	0.00	26	0.00	5	0.00	207	0.00
Potato leafroll virus	55	0.00	28	0.00	3	0.00	4	0.00	5	0.00	95	0.00
<i>Cycas</i> necrotic stunt virus	4	0.00	22	0.00	5	0.00	1	0.00			32	0.00
Melon aphid-borne yellows virus	10	0.00	13	0.00	1	0.00	2	0.00	3	0.00	29	0.00
Pepper vein yellows virus	4	0.00	7	0.00	4	0.00	2	0.00			17	0.00
Tomato aspermy virus	6	0.00	3	0.00	2	0.00			1	0.00	12	0.00
Potato latent virus	2	0.00	4	0.00	2	0.00			1	0.00	9	0.00
<i>Saccharomyces</i> 20S RNA narnavirus	5	0.00	3	0.00							8	0.00
Cucumber green mottle mosaic virus	4	0.00	2	0.00							6	0.00
<i>Saccharomyces cerevisiae</i> virus L-A	5	0.00									5	0.00
<i>Saccharomyces cerevisiae</i> virus L-BC	2	0.00	1	0.00	1	0.00	1	0.00			5	0.00
Blackberry virus E	1	0.00	3	0.00							4	0.00
<i>Mirabilis jalapa</i> mottle virus			3	0.00			1	0.00			4	0.00
Potato virus X	2	0.00	2	0.00							4	0.00

Table 2. Continued.

Virus	J7	%	J13	%	J18	%	J25	%	J29	%	Total	%
Turnip vein-clearing virus	2	0.00	2	0.00							4	0.00
<i>Rehmannia</i> mosaic virus			3	0.00							3	0.00
Shallot virus X	2	0.00	1	0.00							3	0.00
Tomato aspermy virus	3	0.00									3	0.00
Bean common mosaic virus	2	0.00									2	0.00
Peanut stunt virus satellite RNA	1	0.00							1	0.00	2	0.00
<i>Saccharomyces cerevisiae</i> killer virus M1	1	0.00	1	0.00							2	0.00
Tomato spotted wilt virus	1	0.00					1	0.00			2	0.00
Cucurbit chlorotic yellows virus					1	0.00					1	0.00
Peanut stunt virus	1	0.00									1	0.00
Santeuil nodavirus	1	0.00									1	0.00
Scallion virus X	1	0.00									1	0.00
Tobacco vein distorting virus			1	0.00							1	0.00
Tomato necrotic stunt virus	1	0.00									1	0.00
Wasabi mottle virus	1	0.00									1	0.00
Total	7,166,178		5,801,188		799,652		1,179,043		361,863		15,307,924	

A. Pepper mild mottle virus**B. Garlic virus A****Fig. 1.** Sequencing read-depth distributions of *kimchi* PMMoV (A) and GarV-A (B).

Kimchi sample names are shown on the left. Maximum read-depths are shown on the right. ORFs encoded by PMMoV and GarV-A genomes (NCBI Accession No. NC_003630 and NC_003375, respectively) are shown at the top of each panel.

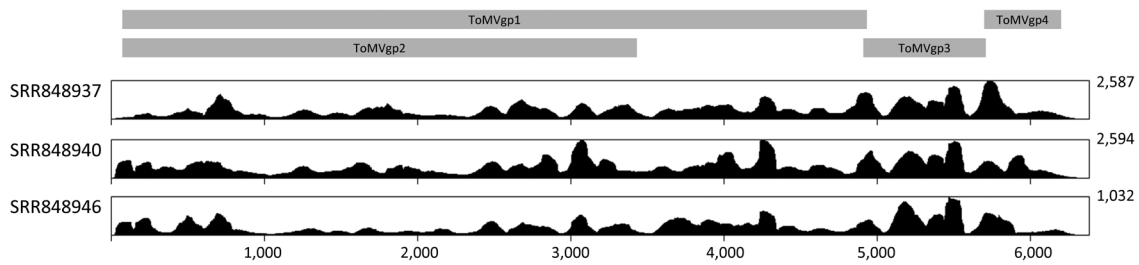


Fig. 2. Sequencing read-depth distribution of ToMV identified from human fecal microbial metatranscriptome data. SRA accession numbers are shown on the left. Maximum read-depths are shown on the right. ORFs encoded by ToMV genome (NCBI Accession No. NC_002692) are shown at the top.

large amount of ToMV sequences were analyzed [6]. Three of the 59 individual data sets contained more than 10,000 sequencing reads derived from ToMV. Although they were prepared independently, the read-depth plots of these three samples showed a similar pattern (Fig. 2). This observation suggested that the RNA viral genome might show a distinct sequencing read-depth distribution.

Sequence of the *Kimchi* PMMoV

To determine the sequence of PMMoV isolated from *kimchi*, a consensus sequence was generated by assembling the PMMoV sequence reads. However, a large variety of PMMoV genotypes might have been simultaneously present in the *kimchi* microbial metatranscriptome that showed short reads ranging from 50 to 100 bp. Therefore, it was impractical to obtain full-length sequences of individual genotypes. Instead, sequences that were different from the reference PMMoV genome sequence NC_003630 (Table S2) were identified. Among the 6,357 nucleotide positions, six sites were different from that of the reference genome; 158 were dimorphic, with one morph being the same as that in the reference; and 56 (15 bp from the 5' end and 41 bp from the 3' end) were not determined. Therefore, a maximally diverged genotype, if present, would show a 2.6% difference from the reference genome (164 differences over 6,301 positions of the determined sequences).

Plant RNA Virus Sequences in Cow Rumen, Human Gut, and Human Fecal Microbial Metatranscriptome Data Sets

Plant RNA virus particles or sequences are frequently detected in the human gut and fecal samples *via* virus particle isolation, RT-PCR, or RNA sequencing [4, 6, 31]. To test the possibility that plant RNA virus sequences are present in other microbial metatranscriptome data, three publicly available data sets derived from cow rumen [22], human gut [16], and human fecal samples were downloaded [26].

These data sets were originally analyzed for microbial gene expression profiles; possible RNA virus presence was not investigated.

BLASTN searches of the reference RNA virus genome database were performed using the three public microbial metatranscriptome data sets as queries (Table 3). The cow rumen microbial metatranscriptome was found to contain plant RNA virus sequences, such as barley yellow dwarf virus, although the number of viral sequences was very small. The human gut and fecal microbial metatranscriptomes also showed some plant RNA virus sequences, including cherry leaf roll virus and PMMoV, further demonstrating that plant RNA viruses are present in the digestive tract of these animals. However, there is no direct evidence that plant RNA viruses are able to infect animal cells. Therefore, it is likely that these plant RNA viruses detected in cow rumen, human gut, and fecal samples were ingested with the infected plant matter.

Interestingly, some animal RNA virus sequences were also detected in human gut and fecal samples; these included leukemia virus, sarcoma virus, respiratory syncytial virus, influenza virus, and parainfluenza virus sequences. This result suggests that the human gut and fecal metatranscriptome data may also be useful for the detection and screening of human RNA viral pathogens.

Possible Indicator of Final Product Quality or the Quality of the *Kimchi* Source Material

The results of this study showed that *kimchi* contains a substantial amount of plant RNA viruses throughout all the stages of fermentation. The RNA virus sequence content was the highest during the early stages of fermentation, and then it declined gradually. It was not clear whether the decline in the RNA virus sequence content was due to the destruction of the RNA virus particles and genomes, or to the overwhelming growth of LAB. Our results suggest that

Table 3. RNA virus sequences present in cow rumen, human gut, and human fecal microbial metatranscriptome data sets.

Sample	Virus	Accession No.	Reads
Cow rumen	Barley yellow dwarf virus-PAS	NC_002160.2	6
	Barley yellow dwarf virus-PAV	NC_004750.1	2
	Brome mosaic virus	NC_002026.1, NC_002028.2	2
	Beet ringspot virus	NC_003694.1	1
Human gut	Cherry leaf roll virus	NC_015414.1, NC_015415.1	27
	Moloney murine leukemia virus ^a	NC_001501.1	24
	Moloney murine sarcoma virus ^a	NC_001502.1	6
	Brassica yellows virus	NC_016038.1	3
	Beet western yellows ST9 associated virus	NC_004045.1	2
Human feces	Pepper mild mottle virus	NC_003630.1	24
	Human respiratory syncytial virus ^a	NC_001781.1	17
	Influenza A virus (A/New York/392/2004(H3N2)) ^a	NC_007367.1	16
	Human parainfluenza virus 2 ^a	NC_003443.1	11
	Paprika mild mottle virus	NC_004106.1	6
	Influenza B virus ^a	NC_002207.1	3
	Strawberry mild yellow edge virus	NC_003794.1	2
	Influenza A virus (A/Puerto Rico/8/1934(H1N1)) ^a	NC_002016.1	1
	Tomato mosaic virus	NC_002692.1	1

^aAnimal viruses.

the detection of plant RNA virus sequences may be an indicator of the quality of the *kimchi* product or source materials, especially red pepper powder and garlic paste [3]. We propose that PMMoV sequenced from red pepper powder samples prepared from a mixture of clean and infected red peppers in different ratios may serve as a quality standard for the red pepper powder in *kimchi*.

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