OPEN ACCESS Freely available online

Journal of Infectious Diseases & Preventive Medicine

Research Article

A Novel *Limosilactobacillus reuteri* Strain Byun-re-01 Exhibits Promising Probiotic Properties for Further Use as Preservatives: An *In Vivo* and *In Silico* Study

Dongjun Kim[1], Ika Nurlaila[1,6], Hanary Geby Jessica Sianipar[1], Mun-Ju Cho[1], Yongjun Lee[1], Seungchan Cho[2], Eui-Joon Kil[3], Sung June Byun[4], Hee Kang[5], Sukchan Lee[1*]

[1]Department of Integrative Biotechnology, Sungkyunkwan University, Suwon, Republic of Korea;[2]Department of Microbiology, College of Medicine, Korea University, Seoul, Republic of Korea;[3]Department of Plant Medicine, Andong National University, Andong, Republic of Korea;[4]Animal Biotechnology Division, National Institute of Animal Science, Rural Development Administration, Wanju-gun, Republic of Korea;[5]Humanitas College, Kyung Hee University, Yongin, Republic of Korea;[6]Department of Vaccine and Drugs Development, The National Research and Innovation Agency, Jakarta, Indonesia

ABSTRACT

Antibiotics had been a pivotal substance to be supplemented in the food for poultry flock with a main purpose to improve their yields in order to gain profits. However, its long-term use costs some adverse effects such as resistance and tolerance. Hence, a new platform is of urgency to figure out. We discovered that our probiotic bacteria, which through the whole-genome sequencing was identified as *Limosilactobacillus reuteri* strain Byun-re-01 (L. *reuteri* Byun-re-01), exhibited promising biological properties to substitute the existing use of antibiotics. Oral administration of L. *reuteri* Byun-re-01 was shown to provide local protection by producing anti-inflammatory cytokines mainly IFN-β and IFN-γ in mRNA levels. In addition, they suppressed the expression of viral capsid mRNA of MNV indicating their antiviral activity. They were not detected to translocate to distant organs but to reside and colonize only within the gut. The ALT/AST levels in the serums of mice receiving L. *reuteri* Byun-re-01-treatment were observed to be at normal range suggesting that no functional damages of the mice's livers were detected. Overall, our results showed favorable profiles of antiviral-carrying probiotics in L. *reuteri* Byun-re-01 that render it a promising candidate of non-antibiotic preservatives.

Keywords: *Limosilactobacillus reuteri* strain Byun-re-01; Probiotic; Safety; Immunomodulatory effect; Anti-murine norovirus; Probiotic-specific markers

INTRODUCTION

Rapid development in the poultry industry has produced high yields in breeding facilities. However, it causes higher immunological stress reactions and worsen the risk of disease outbreaks in commercial poultry flocks [1]. Severe stresses are the primary epidemiological factor that induces immune stress response that further reduces their productivity [2]. This is unacceptable because productivity or yield is the core of purposes. Thus, antibiotics were thought to be the answer for how to maintain the productivity of the commercial poultry [3]. Its use as a supplementary component in the feed was evidenced to improve up to 10% of the daily growth of animals. Furthermore, the meat produced from the antibiotic-fed animals was observed to be richer in protein but lesser in fat compared to that fed with no antibiotic [4]. However, continuous use of antibiotic has arisen a major concern, that is the antibiotic-resistance and tolerance which would hamper pathogen eradication during infection. These two, thus far, are a persistent health care problem globally, hence other platforms than antibiotics are urged to be discovered [3].

An interesting report by Jose, et al. showed that rumen-origin probiotic bacteria exhibited three excellent features: 1) high tolerance to stress conditions, 2) high inhibition of pathogen growth, and 3) high adherence property [2]. The rumen-origin bacteria co-exist with pathogens in the intestine thus competitive relationship between the two populations is maintained rendering the rumen-origin bacteria display a considerably high inhibition

Correspondence to: Sukchan Lee, Department of Integrative Biotechnology, Sungkyunkwan University, Suwon, Republic of Korea, Tel: +82-31-290-7866; Fax: +82-31-290-7870; E-mail: cell4u@skku.edu

Received: 04-May-2022, Manuscript No. JADPR-22-17330; **Editor assigned:** 09- May -2022, PreQC No. JADPR-22-17330 (PQ); **Reviewed:** 23-May-2022, QC No. FGB-22-17330; **Revised:** 27-May-2022, Manuscript No. JADPR-22-17330 (R); **Published:** 03-Jun-2022, DOI:10.35841/2329-8731.22.10.271.

Citation: Kim D, Nurlaila I, Sianipar HGJ, Cho M, Lee Y, Cho S, et al. (2022) A Novel *Limosilactobacillus reuteri* Strain Byun-re-01 Exhibits Promising Probiotic Properties for Further Use as Preservatives: An *In Vivo* and *In Silico* Study. J Infect Dis Preve Med. 10: 271.

to the pathogen growth than that shown by dairy-origin isolates [2]. This inspired us to dig deeper in the probiotic bacteria-realm in order to discover novel rumen-origin bacteria which were potentially advantageous to address huge troublesome faced in the poultry productivity, particularly in improving the overall growth and quality of the products, which was expected to outperform antibiotics use.

Some bacteria species such as *Limosilactobacillus reuteri* (L. *reuteri*), *Lactobacillus johnsonii (L. johnsonii), Lactobacillus gasseri (L. gasseri), Limosilactobacillus fermentum (L. fermentum),* and *Lactobacillus plantarum (L. plantarum)* are well known for their probiotics properties [5]. They are reported to be capable of inhibiting rotavirus infection by magnifying the expression of interferon-α (IFN-α), IFN-β, and IFN-induced antiviral effectors such as myxovirus resistance-A, 20,50-oligoadenylate synthetase, and protein kinase R [6]. The bacteria are commonly harnessed as a medicament to treat patients with diarrhea and inflammatory bowel disease [7]. However, in immunocompromised patients, instead of eliciting protection mechanisms which favor the patients' health, the bacteria induce rare infections such as bacteremia, particularly caused by *Lactobacillus*, and endocarditis that is ascribed to Bifidobacterium [8,9]. Therefore, it is critical to secure the safety before utilization, especially if the bacteria inhabit within the digestion system of human or animal [10]. Up to date, norovirus-induced digestion system diseases are not licensed to any specific treatment which speaks more volume in the urgency to pick it as our focus in this particular study [11]. Unfortunately, as *in vitro* setting of human norovirus is yet to be available however, a standardized *in vitro* instrument to investigate human norovirus was and is yet to be available thus a surrogate system which mimics this at best is necessitated [12]. In this purpose that murine norovirus (MNV) emerges as an excellent substitute [13-15].

There have been a few reports showed that Bifidobacterium, *Lactobacillus*, and Lactococcus, were capable of conferring resistance against MNV [15]. A couple years later we displayed similar features where our *L. paracasei* ATCC 334, which was prior-engineered to express 3D8 single-chain variable fragment (scFv), elicited antiviral activities against MNV. In addition, a report from Cho et al. demonstrated that the probiotic activity of the *L. paracasei* ATCC 334 was the primary cause for the bulking-up population of *Pediococcus acidilactici* and the lowered population of Helicobacter in the mice intestines [16]. This specific result has driven us to an initial yet critical understanding that the *L. paracasei* ATCC 334 was a promising probiotic candidate. However, the survival rate of the *L. paracasei* ATCC 334 might be a major drawback for its utilization because the bacteria were observed to be having a low survival rate where practically they were expected to highly survive. Furthermore, as their colonies were considerably short-lived, this adds more to the hindering aspect of exploiting *L. paracasei* ATCC 334 as an antiviral-eliciting probiotic. This led us to a novel finding of murine intestine-isolated probiotic bacteria which was further identified and classified as *Limosilactobacillus reuteri* strain Byun-re-01 (L. *reuteri* Byun-re-01). At last, we performed whole-genome and transcriptome sequencing to evaluate biosafety markers of the L. *reuteri* Byun-re-01. Altogether, our results can serve as a strong basis for the use of probiotic-potential bacteria as preservatives which might promote or improve immune responses of the host against viral-attacks.

MATERIALS AND METHODS

Bacterial isolation and culture conditions

Six-week-old, specific pathogen-free ICR mice (DBL, Eumseong, Korea) were sacrificed before isolating *Lactobacillus* from their small intestines. The small intestines were cut into three parts: duodenum, jejunum, and ileum. The extracted tissues were homogenized with 1.6 mm stainless steel beads (Next Advance, NW, USA) and phosphate-buffered saline (PBS). The homogenized tissues were spread on Man-Rogosa-Sharpe (MRS) plates (Merck Millipore, Boston, MA, USA), to grow various *Lactobacillus* species. We randomly selected a colony among the colonies grown on the MRS plate. Only bacteria that were rod-shaped under microscopy were selected and cultured anaerobically in MRS broth for two days at 37°C. The isolated bacteria were maintained in MRS medium supplemented with 80% glycerol. As a positive control, the probiotic strains *Lacticaseibacillus paracasei* ATCC 334 and *Limosilactobacillus reuteri* KACC 11452, which were provided by the courtesy of Dr. Jos Seegers (Falcobio, Leiden, Netherlands) and the Rural Development Administration (Jeonju, Korea), respectively.

Primer design

All primers used in this study are listed in Table 1 [17]. The 9F and 1542R primers were used to determine bacterial species using 16S ribosomal DNA (rDNA) as previously reported [18]. DNA gyrase subunit B (gyrB) was used to retrieve the sequences of several *Lactobacillus* species from GenBank (www.ncbi.nlm.nih.gov) to perform multiple alignments *via* MultAlin. The DNA gyrB primer was designed for the region of the gyrB sequence most conserved among several *Lactobacillus* species. The sets of cytokine primers and MNV detection primers were designed using the Primer 3 program.

Table 1: All primers used in this study.

Gene	Forward (5'→3')	Reverse (5'→3')	Reference
16S rDNA	GAGTTTGATCCTGGCTCAG	AGAAAGGAGGTGATCCAGCC	[17]
DNA gyrase subunit B	GCGGAAGCGGCCNGSNATGTA	CCGTCCAGCTCGGCRTCNGYCAT	This study
GAPDH	TGGCAAAGTGGAGATTGTTGCC	AAGATGGTGATGGGCTTCCCG	NM_002046
IFN-β	TTACACTGCCTTTGCCATCCAA	TCCCACGTCAATCTTTCCTCTT	NM_010510.1
IFN-γ	ACTGGCAAAAGGATCGTGAC	GACCTGTGGGTTGTTGACCT	NM_008337.3
MNV capsid protein	CTCTCAGCCATGTACACCGG	TAGGGTGGTACAAGGGCAACAA	JQ237823.1

Identification of *limosilactobacillus*

Two conserved genes, 16S rDNA and DNA gyrB, were used to identify bacterial species. Purification of each PCR product was conducted using a Zymoclean™ Gel DNA Recovery Kit (Zymo Research, CA, USA), followed by Sanger/capillary sequencing (Macrogen, Seoul, Korea). Bacterial species were identified using the EzTaxon-e database with 16S rDNA data and BLAST analysis of the gyrB data [18].

Phylogenetic analysis

Gene sequences of 16S rDNA and gyrB were retrieved from GenBank (www.ncbi.nlm.nih.gov). Multiple alignments were next performed by using MEGA program (version 7). Parallelly, to construct phylogenetic trees between L. *reuteri* Byun-re-01 and other L. *reuteri* strains namely *L. rhamnosus* GG, *L. fermentum* IFO 3956 and *L. paracasei* ATCC 334, we applied neighbor-joining method with bootstrap values (1,000 replicates) in MEGA program.

Whole-genome sequencing, assembly, and annotation

Whole-genome sequencing of L. *reuteri* Byun-re-01 was performed following published methods [19]. Bacterial genomic DNA was extracted using a G-spin™ Genomic DNA Extraction Kit (iNtRON, Sungnam, Korea) following to the manufacturer's protocols. The sequencing was performed *via* two sequencing platforms: PacBio RS II (Pacific Biosciences, CA, USA) and Illumina Hiseq 2000 (Illumina, Inc., CA, USA), at Macrogen (Seoul, Korea). The construction of the complete genome of L. *reuteri* Byun-re-01 was done in a two-step assembly process. First, de novo assembly of the PacBio raw sequencing data was conducted using Single Molecule, Real-Time (SMRT) Analysis software version 2.3.0 [20]. Second, hybrid assembly of the Illumina raw sequencing data was performed using Pilon (v1.21) to increase the accuracy of the assembly. The complete genome was annotated using Prokka software version 1.12b. The resulting complete genome of L. *reuteri* Byun-re-01 was registered at the Pubmed's GenBank with an accession number CP029613.

Comparative genomic analysis

By using the data from the NCBI genome database (http://www.ncbi.nlm.nih.gov/genome), nine L. *reuteri* strains were selected based on the phylogeny results (L. *reuteri* strains DSM 20016, IRT, JCM 1112, I49, TD1, ATCC 53608, ZLR003, I5007, and SD2112) and compared with the whole genome of L. *reuteri* Byun-re-01. The OrthoANI algorithm was used to compare the similarity of the ten genomes to determine whether the isolated strain was a novel type [21]. A phylogenetic tree was constructed based on the OrthoANI values of the L. *reuteri* strains.

Mice, virus, and cells

Six-week-old female ICR mice (DBL, Eumseong, Korea) were used as our resource to isolate the *Lactobacillus* strains from. On the other hand, 6 weeks of female C57BL/6 mice were employed to assay antiviral activity of *Limosilactobacillus* isolates against MNV strain CR6 (MNV CR6). All mice were acclimatized for one week prior to the main experiment. The mice were housed following a standardized laboratory condition. RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA) containing 10% heat-inactivated fetal bovine serum (FBS) (Alphabioregen, Boston, USA), 100 units/ml of penicillin, 100 µg/ml streptomycin (Pan Biotech, Aidenbach, Germany), 10 mM HEPES, and 2 mM glutamine in a 5% CO_2 incubator at 37°C. MNV CR6 was provided by the courtesy of Dr. Herbert W. Virgin from Washington University and proliferated in RAW264.7 cells [22].

Selection tool for evaluating survival rate of *Limosilactobacillus*

A pSLP111.3 vector was provided by Dr. Jos Seegers (Falcobio, Leiden, Netherlands). It was prior-modified by replacing the xylose-inducible promoter with a lactate dehydrogenase constitutive promoter and transformed into *Limosilactobacillus* and *Lacticaseibacillus* was performed as previously described [15]. These transformed bacteria were, then, used to measure the retention time of *Limosilactobacillus* and *Lacticaseibacillus* in the Gastrointestinal Tract (GIT) of mice as previously described [23].

Preparation of *Limosilactobacillus* strain for administration

Bacterial seeds were inoculated with MRS medium at a 1:50 ratio and incubated for 16 hours at 37°C. After diluting the culture to the OD600: 1, the culture was subjected to 10-fold serial dilutions and spread on the MRS plate. The number of colonies forming units (CFUs) of *Limosilactobacillus* and *Lacticaseibacillus* strains contained in 1 ml of medium was calculated by the plate counting method. Based on that CFU value, 108 CFUs were harvested and prepared.

Persistence of *Limosilactobacillus* strain in the GIT of mice

To determine the retention time of the L. *reuteri* Byun-re-01 in murine intestines, mice were assigned to several experimental groups as follows: 1) the presence or absence of antibiotic pretreatment before the experiment and 2) the number of feedings. Three ICR mice were assigned to each of the four experimental groups. To allow for retention of the pSLP111.3 vector and remove partially commensal bacteria in the intestine, all mice were given water containing 3 µg/ml chloramphenicol for 24 h. Food and water were removed for 18 h before the probiotic feeding. The mice were fed 108 CFUs of *L. paracasei* ATCC 334 and L. *reuteri* Byun-re-01 for several assignments: only once or once every two days, over six days. In the experimental group that did not receive antibiotics, the mice were fed 108 CFUs of bacteria. After feeding, food and water containing antibiotics were given to the mice again. Feeding needles (20 gauge) were used for all oral administration procedures. Feces (1 g) were collected daily from each cage and resuspended in PBS, followed by serial dilution. Diluted fecal samples were plated on MRS plates supplemented with 3 µg/ml of chloramphenicol and incubated anaerobically at 37°C for two days. In the negative control group, mice were fed PBS to match the other experimental conditions. CFUs were calculated using a plate counting method.

Assessment of bacterial translocation

The translocation of bacteria from the gut to the blood and other tissues was assessed as previously described [24]. The liver and kidneys were harvested aseptically, and each organ was homogenized. About 100 µl of homogenates and 20 µl of blood were plated on MRS agar and Brain Heart Infusion agar (BHI) (BD Biosciences, San Jose, CA, USA). Next, the plates were incubated

for 48 hours at 37°C.

Gut-associated immunomodulation by *Limosilactobacillus* strain

Total RNA from intestine tissues was extracted using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). cDNA was synthesized from 2 μg of total RNA with oligo dT primer and Moloney murine leukemia virus reverse transcriptase (Bioneer, Daejeon, Korea) following the manufacturer's protocols. To quantify *Limosilactobacillus*- and *Lacticaseibacillus* strains-induced cytokine levels, Rotor-Gene Q system-based quantitative real-time PCR (Qiagen, Chadstone, Victoria, Australia) was performed. SYBR Premix Ex Taq (TaKaRa, Kyoto, Japan) was used for PCR amplification and intercalator. Data were analyzed using Rotor-Gene Q series software version 2.3.1 (Qiagen).

Quantification of MNV titer

A plaque-based assay was performed as previously described to determine the virus concentration [25]. We used 0.5% crystal violet (Sigma-Aldrich, St. Louis, USA) for plaque visualization after fixation with 4% formaldehyde (Sigma-Aldrich). A negative control well was added per plaque assay.

Antiviral activity test Against MNV CR6

Six-week-old, MNV-seronegative female C57BL/6 mice were used to evaluate the anti-MNV activity of L. *reuteri* Byun-re-01. Its antiviral activity was compared with that of the *L. paracasei* ATCC 334 strain and the L. *reuteri* KACC 11452 strains derived from humans. Mice were fed 108 CFUs of wild-type L. *reuteri* Byun-re-01, L. *reuteri* KACC 11452, or *L. paracasei* ATCC 334 for two weeks, followed by infection with 105 plaque-forming units (PFUs) of MNV. Stool sampling was performed on days 5, 7, 9, and 14 after viral infection. Fecal RNA was extracted using a Quick-RNATM Fecal/Soil Microbe Microprep kit (Zymo Research, CA, USA). cDNA was synthesized using CellScript cDNA synthesis master mix (CellSafe, Yongin, Korea), and viral titers were compared among experimental groups using quantitative real-time PCR in the same manner as described above.

RNA extraction and transcriptome sequencing

After being cultured at pH 4.8, L. *reuteri* Byun-re-01 were lysed using lysozyme (20 mg/ml) and mutanolysin (50 U/ml) (Sigma Aldrich, St. Louis, MO, USA) treatment and subjected to the total RNA extraction using NucleoSpin® RNA kit (MACHEREY-NAGEL, Düren Germany). Following treatment with DNase to prevent DNA contamination of the total RNA samples, mRNA purification was performed using Ribo-Zero rRNA Removal Kit (Bacteria) (Illumina, Inc., CA, USA). A TruSeq RNA Sample Prep Kit v2 was then used according to the manufacturer's instructions for library preparation. Paired-end libraries with 200bp–400bp insert sizes were sequenced using a HiSeq2000 (Macrogen, Seoul, Korea).

Calculation mapping and fragments per kilobase of transcript per million mapped reads

The raw data (4 fastq files) generated from the Illumina sequencing were deposited in GenBank under accession number SRR8988597. Trimmed reads were resulted fromTrimmomatic 0.32 software to remove the adaptor sequence. Those reads were mapped on the reference sequence (the genome of L. *reuteri* Byun-re-01) using BBMap (short-read aligner) with default parameters. After the mapping process, data mining to find probiotic-specific markers was performed [26].

Statistical analysis

Our data point was arranged as triplicates and presented as means ± SEM in GraphPad Prism (GraphPad software version 5). One way ANOVA was performed, and significance was determined by the P value<0.05.

RESULTS

Isolation and identification of *Limosilactobacillus* strains from mouse small intestines

We screened more than 200 colonies from duodenal tissues and selected 80 rod-shaped bacteria for further strain identification using 16S rDNA sequences. Most isolates were identified as L. *reuteri* strains, with an exception to a few species such as *Lactobacillus* intestinalis and putative *Lacticaseibacillus paracasei*. This analysis also resulted in 99.47% similarity to that seen in L. *reuteri* JCM 1112. In addition, we also performed characterization using gyrB which resulted in 99.64% similarity to that of L. *reuteri* strain I49. Given these results from both 16S rDNA and gyrB analysis, we only took L. *reuteri* isolates for further analysis.

Phylogenetic analysis bsed on conserved genes of L. *reuteri* strains

The aligned sequences, which had an average of 1,500 bp and 1,900 bp of 16S rDNA and gyrB sequences, respectively, were analyzed to determine the phylogenetic relationships. Phylogenetic trees were constructed based on the neighbor-joining method using bootstrap values (1,000 replicates) (Figure 1). They showed that the L. *reuteri* mouse isolates were genetically in proximity to various L. *reuteri* strains.

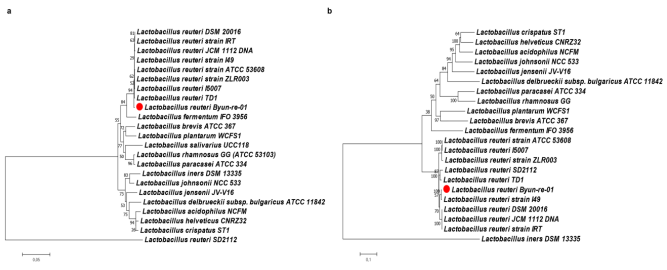

Figure 1: Phylogenetic tree based on comparison of 16S rDNA and gyrB sequences of L. *reuteri* mouse isolates and reference sequences. The phylogenetic trees were generated using the neighbor-joining method with an average of 1,500 aligned nucleotides in 16S rDNA (A) and gyrB (B). Bootstrap values (from 1,000 replicates) are shown at the branch points. Most values were greater than 40%. The *Limosilactobacillus*, *Levilactobacillus*, *Lactiplantibacillus*, *Ligilactobacillus*, *Lacticaseibacillus*, and *Lactobacillus* species used to determine genetic relationships included: various L. *reuteri* strains, *L. fermentum*, *L. crispatus*, *L. brevis*, *L. paracasei*, *L. salivarius*, *L. plantarum*, *L. rhamnosus*, *L. iners*, *L. johnsonii*, *L. jensenii*, *L. acidophilus*, *L. delbrueckii*, and *L. helveticus*. The scale represents 0.1 nucleotide substitution per position.

Analysis of the genomic features of the isolated *Limosilactobacillus* strain

As delineated in the bacteria screening results, most of the isolated bacteria were identified as L. *reuteri*. As we needed to evaluate that the bacteria were probiotic-potential, we performed whole genome sequencing analysis using general genomic features detailed in Table 2. This generated a strain map of L. *reuteri* Byun-re-01 (Figure 2). A total of 68,530,004 paired end reads generated from the Illumina sequencing and 128,652 long reads generated from the PacBio sequencing were employed to construct the complete chromosome. The complete genome of L. *reuteri* Byun-re-01 was 2,244,514 bp long (N50 values 2,244,514 bp) with 38.9% GC content constituted the genome. This genome contained 18 rRNA genes, 72 tRNA genes, and 2,083 protein-coding sequences. Furthermore, we identified the distribution of clusters of orthologous groups (COGs) from the genome.

Table 2: General genome features of L. *reuteri* Byun-re-01.

	L. *reuteri* Byun-re-01
Sequencing platforms	PacBio RS II / Illumina Hiseq2000
Assembler	PacBio SMRT Analysis 2.3.0 / Pilon (v1.21)
Number of reads	128,652 (PacBio) / 68,530,004 (Illumina)
Genome coverage	404
Genome size (bp)	22,44,514
G+C content (%)	38.9
Predicted CDS	2,083
Number of contigs	1
Number of rRNA genes	18
Number of tRNA genes	72
N50 (bp)	22,44,514

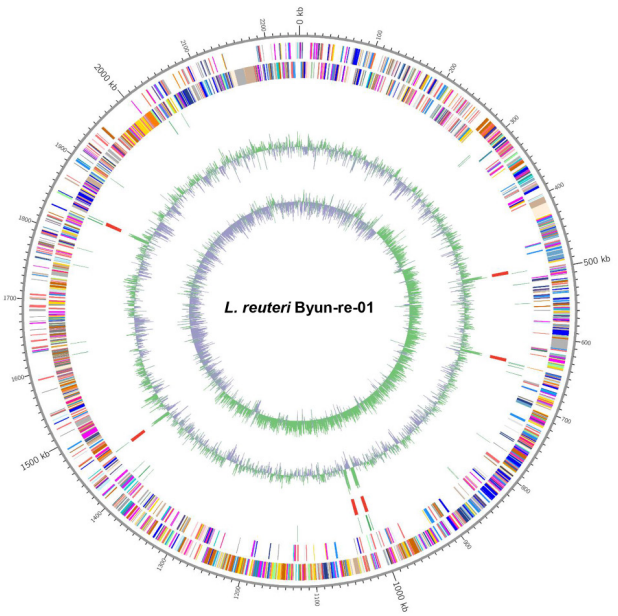

Figure 2: Circular representation of the L. *reuteri* Byun-re-01 genome. Circular map of the *Limosilactobacillus reuteri* strain Byun-re-01 genome. Marked characteristics are shown from outside to the center; CDS on forward strand, CDS on reverse strand, tRNA (light green), rRNA (red), GC content, and GC skew. This map was generated by circos.

In order to check if our newly discovered bacterial strain was novel, we compared our whole-genome results with top nine closest reference strains (Figure 3). A phylogenetic tree was constructed based on the average nucleotide identity (ANI) values. L. *reuteri* Byun-re-01 had more than 95% similarity to the other L. *reuteri* strains that were previously accessioned in NCBI database (Figure 4). It solidified the novelty of L. *reuteri* Byun-re-01. This particular strain was then registered to NCBI genome database with accession number CP029613.

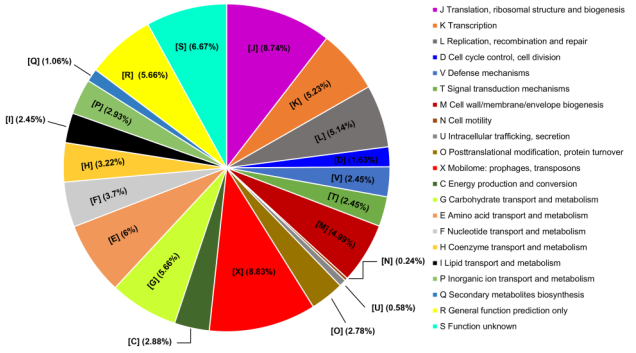

Figure 3: Analysis of clusters of orthologous groups (COG) functional categories. Abundance of COG functional categories in L. *reuteri* Byun-re-01. Coding sequences were identified using PSI-BLAST through NCBI COGs, 2014 version (http://www.ncbi.nlm.nih.gov/COG/), based on sequence homology.

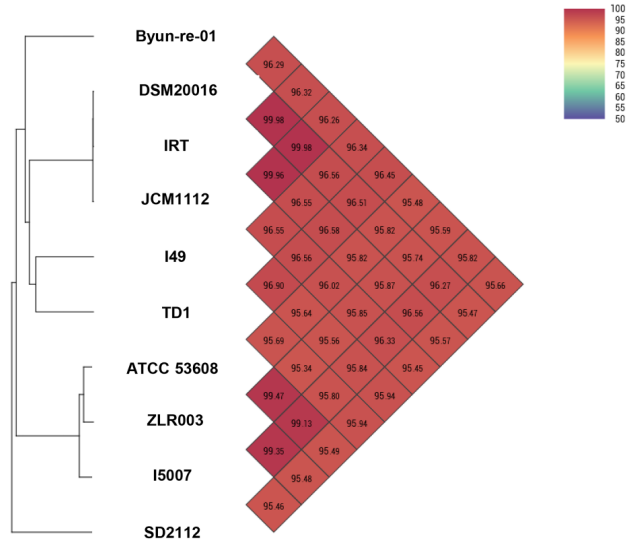

Figure 4: Comparison of whole-genome sequence among *Limosilactobacillus* strains through comparative genome analysis. An ANI phylogenetic tree was constructed based on OrthoANI values. Similarities between two genomes are indicated next to the phylogenetic tree.

Persistence of *Limosilactobacillus* strain in the gastrointestinal tract

Probiotics should possess resistance to low pH to survive and colonize in the gastrointestinal tract (GIT). The chloramphenicol resistance gene and lactate dehydrogenate constitutive promoter, which was inserted into the pSLP111.3 expression vector Figure 5, were harnessed to positively select intestinal low pH-resistant

strains of L. *reuteri* Byun-re-01, to represent *Limosilactobacillus*, and *L. paracasei* ATCC 334, to represent *Lacticaseibacillus* [15]. Our data show that the survival rate of L. *reuteri* Byun-re-01 was higher than that of *L. paracasei* in all doses we gave (Figures 6A and 6B). Furthermore, in the absence of antibiotic, L. *reuteri* Byun-re-01 remained in the GIT longer than *L. paracasei*. However, all strains survived shorter compared to that seen in antibiotics-treated mice counterpart (Figures 6C and 6D).

Safety assessment of L. *reuteri* byun-re-01 strain

To assess whether or not the isolated L. *reuteri* Byun-re-01 safe for consumption, this strain was orally administered to mice for two weeks. As comparison, we had *L. paracasei* ATCC 334, to represent *Lacticaseibacillus* strains and control. We did not observe significant change in the daily food intake in *L. paracasei* ATCC 334-treated, L. *reuteri* Byun-re-01-treated groups, even in the control group. However, in terms of increment volume of food intake, L. *reuteri* Byun-re-01 group was in closer range to that seen in control group (Figure 7 and Table 3). In addition, significant disparities, including weight, size and histological outlooks, neither was observed macroscopically in livers, intestines and spleens (Table 4). These suggest that L. *reuteri* Byun-re-01 did not carry adverse-potential impact.

Bacterial translocation assessment

Bacterial translocation is defined as an event in which the administered bacteria migrated from the intestinal tissue into the blood or other organs. We did not observe bacteria translocation from the gut to other tissue in negative control and L. *reuteri* Byun-re-01 treated groups (Table 5). In the case of the mice receiving *L. paracasei* ATCC 334 treatment, we identified one mouse having bacterial translocation in the liver (Table 5). As to detect if livers experienced damage or injury, we measured ALT/AST in the serum of mice treated with *L. paracasei* ATCC 334–treated, L. *reuteri* Byun-re-01 and control. We figure out that the AST/ALT levels in all the groups were within the normal ranges (AST 54–298 U/L, ALT 17–77 U/L). Serum AST levels were detected at the levels of 110, 105, and 102 (U/L) in negative control, *L. paracasei* ATCC 334–treated, and L. *reuteri* Byun-re-01–treated mice respectively; while serum ALT levels for the respective treatments were 38, 44, and 52 (U/L).

Figure 5: Schematic representation of the vector used to determine the retention time of L. *reuteri* Byun-re-01 in the small intestine.

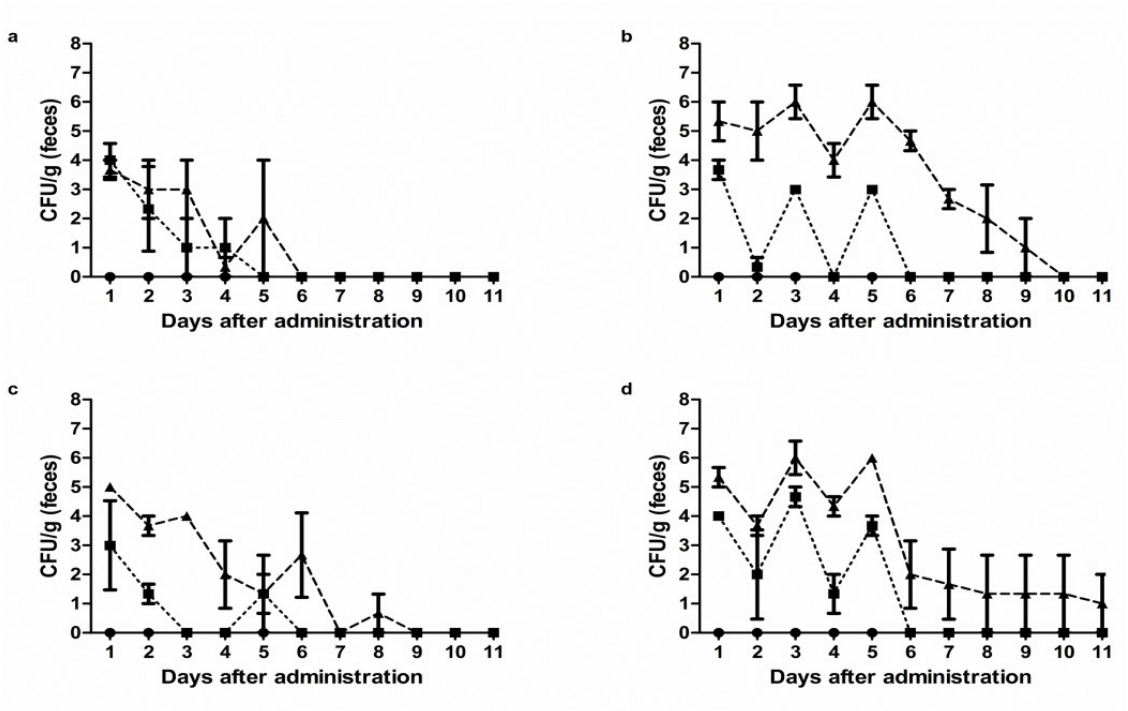

Figure 6: Intestinal colonization of *Limosilactobacillus reuteri* Byun-re-01 as a probiotic under various conditions. (A) Administration of 108 CFUs of *L. paracasei* ATCC 334 and L. *reuteri* Byun-re-01 once with antibiotic pretreatment. (B) Administration of 108 CFUs of *L. paracasei* ATCC 334 and L. *reuteri* Byun-re-01 three times (every two days) with antibiotic pretreatment. (C) Administration of 108 CFUs of *L. paracasei* ATCC 334 and L. *reuteri* Byun-re-01 once without antibiotic pretreatment. (D) Administration of 108 CFUs of *L. paracasei* ATCC 334 and L. *reuteri* Byun-re-01 three times (every two days) without antibiotic pretreatment. Data are presented as the means ± SEM. **Note:** (●) Negative control, (■) *L. paracasei* ATCC 334, (▲) L. *reuteri* Byun-re-01.

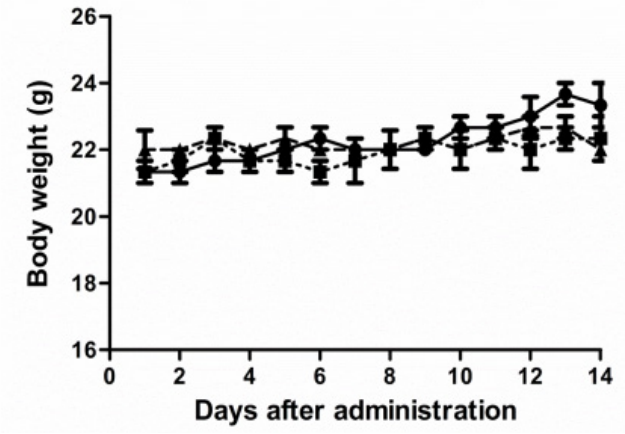

Figure 7: Comparison of weight changes in mice treated with *Limosilactobacillus* and *Lacticaseibacillus* strains. The *L. paracasei* strain ATCC 334 (as a positive control) and L. *reuteri* Byun-re-01 were fed to mice daily for two weeks. The body weights of the mice treated with the *Limosilactobacillus* and *Lacticaseibacillus* strains were measured during the administration period. Data are presented as the means ± SEM for n=3. **Note:** (●) Negative control, (■) *L. paracasei* ATCC 334, (▲) L. *reuteri* Byun-re-01.

Table 3: Measurement of food intake during administration of *Limosilactobacillus* and *Lacticaseibacillus* strains.

Strain	Food intake (g)
Negative control	7.88 ± 0.65
L. paracasei ATCC 334	7.27 ± 1.04
L. *reuteri* Byun-re-01	7.66 ± 0.74

Note: Food intake of mice fed bacteria was measured once every two days. Data are presented as the means ± SEM for n=3.

Table 4: Organ weight measurements of mice fed *Limosilactobacillus* and *Lacticaseibacillus* strains.

Group	Liver (g)	Intestine (g)	Spleen (g)
Negative control	1.27 ± 0.01	0.67 ± 0.02	0.1 ± 0.01
L. paracasei ATCC 334	1.24 ± 0.04	0.65 ± 0.05	0.11 ± 0.02
L. *reuteri* Byun-re-01	1.28 ± 0.21	0.62 ± 0.02	0.08 ± 0.03

After the administration period, mice were sacrificed, and their livers, small intestines, and spleens were harvested. There were no significant differences in all groups. An approximately 10 cm section of small intestine was excised and weighed. Data are presented as the means ± SEM for n=3.

Table 5: Incidence of bacterial translocation in mice fed *Limosilactobacillus* and *Lacticaseibacillus* strains.

	Bacteria	Blood	Kidney	Liver
MRS agar	Negative control	0/3	0/3	0/3
	L. paracasei ATCC 334	0/3	0/3	1/3
	L. *reuteri* Byun-re-01	0/3	0/3	0/3
BHI agar	Negative control	0/3	0/3	0/3
	L. paracasei ATCC 334	0/3	0/3	0/3
	L. *reuteri* Byun-re-01	0/3	0/3	0/3

Animals with tissues from which surviving bacterial cells were recovered were defined as bacterial translocation–positive animals. The numbers in this table are (positive animals/all test animals).

Immune-boosting activity by L. *reuteri* strain byun-re-01

Lactic acid bacteria are generally known to have health-promoting effects in animal models and clinical trials [27]. Mice treated with L. *reuteri* Byun-re-01 magnify their mRNA expressions of IFN-β and IFN-γ to 19.8- and 8.8-fold higher, respectively, relative to that of their negative control counterpart. These expressions were also observed to be increasing to 7- and 9.8-fold when relatively compared to *L. paracasei* ATCC 334 and L. *reuteri* KACC 11452, respectively (Figure 8). These results are consistent with the widely known about lactic acid bacteria capable of promoting over-expressions of innate immune system-associated cytokines.

Antiviral effect of L. *reuteri* strain byun-re-01 against murine norovirus cr6

To evaluate if L. *reuteri* Byun-re-01 carried protection effect for the mice against MNV infection, three wildtype *Limosilactobacillus* and *Lacticaseibacillus* strains, including L. *reuteri* Byun-re-01, were orally administered to the mice daily for two weeks prior to viral infection. Due to the chronic nature of viral infection in this study, antiviral efficacy was assessed in stool samples at days 5-, 7-, 9-, and 14 post-infections. Compared to the infected mice without probiotic treatment, the *L. paracasei*-treated infected mice showed an approximately 2.1-fold reduction in viral capsid mRNA expression over time (Figure 9). We, next, assessed the antiviral effect in human- and mice-derived L. *reuteri* as we presumed that lactic acid bacteria originated from the same organism as the host would have a better impact. Our results demonstrated that the mice treated with human-derived L. *reuteri* KACC 11452 had a 2.3-fold reduction in viral mRNA compared to that of untreated infected mice-counterpart. As expected, mice-derived L. *reuteri* Byun-re-01-treated mice had a 6.6-fold reduction at all sampling periods (Figure 9). These suggested two things. First, L. *reuteri* Byun-re-01 carried better protection activity against MNV, than did *L. paracasei* and L. *reuteri* KACC 11452. Second, the protection was heightened when the bacteria were isolated from the same species as where they were going to be applied.

Identification of probiotic markers based on transcriptome screening

In general, information regarding the number of total RNA reads for L. *reuteri* Byun-re-01 was obtained using next-generation sequencing (Table 6). After removing the adapter sequence using Trimmomatic 0.32 software, we obtained mapped RNA reads using BBmap and applied them for further analysis. Probiotic-related markers of L. *reuteri* Byun-re-01 (cultured at pH 4.8) were identified using transcriptome analysis. The probiotic-related genes were retrieved from published reports [28-33]. In general, probiotics and hosts interact symbiotically. The health-promoting effect of probiotics, which is defined as a probiotic factor, and an adaptation factor of probiotics inside the hosts, can be expressed as virulence factors [29]. The genome of L. *reuteri* Byun-re-01 showed virulence-related factors related to adhesion through data mining, namely a sortase, dltD, dltA (D-adenylation of LTA), hemolysin III, and fibronectin-binding protein (Figure 10). Specifically, we identified exopolysaccharides, important probiotic effector molecules involved in biofilm formation that facilitate the colonization of lactic acid bacteria in the intestines. L. *reuteri* Byun-re-01 also harbored general stress adaptation proteins, chaperone GroES, GroEL, DnaK, DnaJ, and F0F1 ATP synthase subunits. The level of expression of each gene was gauged as fragments per kilobase of transcript per million mapped reads. Our results demonstrated that the expression levels of most genes were high as illustrated in Figure 10. These suggested that L. *reuteri* Byun-re-01, derived from the original murine host, could more efficiently colonize in the intestines and persisted for a longer period in mice than that of bacteria originated from foreign organisms. In addition this provided beneficial effects to the host.

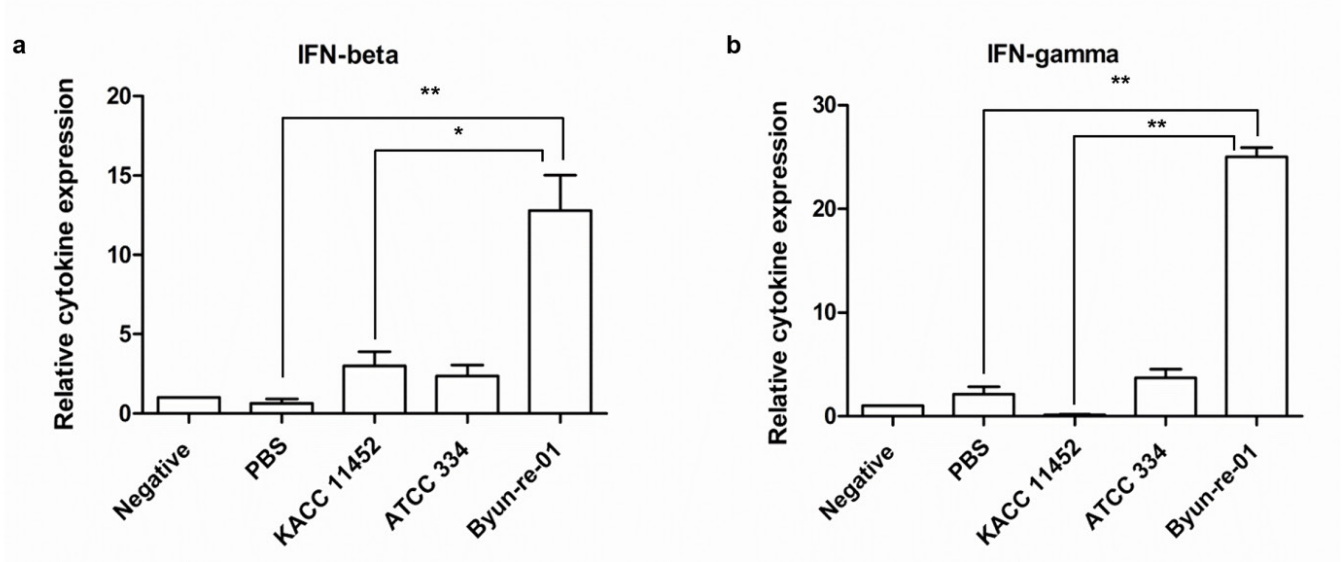

Figure 8: Immune boosting activity of L. *reuteri* Byun-re-01 in gut tissues. Cytokine induction by *Limosilactobacillus* and *Lacticaseibacillus* strains treatment in mice intestine was investigated by measuring the IFN expression. The mRNA expressions of (A) IFN-β and (B) IFN-γ were analyzed using qRT-PCR and relative expression of these genes were quantified relative to the GAPDH mRNA expression using the ΔΔCT method. Data are presented as the means ± SEM for n=3. *p<0.05, **p<0.01.

Figure 9: Comparison of antiviral effect against murine norovirus CR6 among mice treated with *Limosilactobacillus* and *Lacticaseibacillus* strains. Relative expression of MNV capsid protein in stool samples of MNV-infected mice without pretreatment and pretreated with *L. paracasei* ATCC 334, L. *reuteri* KACC 11452, and L. *reuteri* Byun-re-01. GAPDH was used as an internal control, and the relative mRNA level of capsid protein was calculated using the ΔΔCT method. Data are presented as the means ± SEM for n=3. **p<0.01, ***p<0.001. **Note:** (▭) Negative control, (▨) MNV infection, (▭) *L. paracasei* ATCC 334 (▩) L. *reuteri* KACC 11452, (▩)L. *reuteri* Byun-re-01

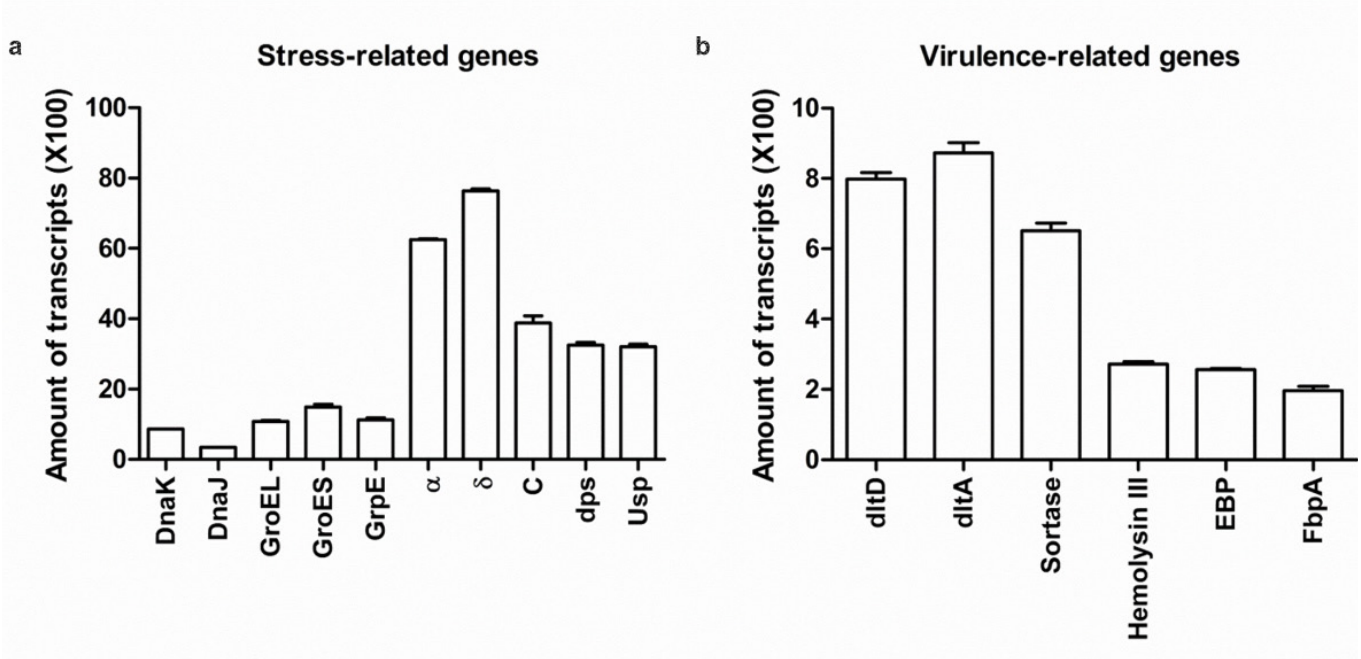

Figure 10: Genome-wide screening for probiotic-related genes. Transcriptome analysis was performed to identify probiotic-related genes. The level of expression of each gene is expressed as fragments per kilobase of transcript per million mapped reads. Data are presented as the means ± SEM for n=2.

DISCUSSION

In the early 20th century, probiotic bacteria were, for the first time, identified by Elie Metchnikoff who discovered particular strains of human gut-residing bacteria that were beneficial to preserve the homeostatis of the gut. Later these bacteria were referred to as probiotics [34]. Soon after, probiotics became a special field in microbiology and attentions toward this intensified following discoveries on new strains of prior-classified bacteria or new bacteria at all which was deemed to be promising to be used in a wide spectrum of commercial commodities within which poultry flock, that undeniably reaches out the livelihood of human being urge a comprehensive improvement in regard with assuring strategy for high-quality and high yield of poultry-related products. On this account, our present study sought to discover novel probiotic bacteria isolated from small intestines of mice that were potential and safe to be harnessed as food preservatives to support the need of high yield-poultry flock for commercial purposes. The probiotic candidates were expected to carry anti-viral activity to enhance the immune response following consumption. When these two features are seen in a candidate (bacterial isolate), that would make them a complete package of a food preservative. That being the case that studies on probiotic bacteria has been bulking up these past few decades [35,36]. Our laboratory has recently accessioned a novel finding of a probiotic strain which was named after L. *reuteri*-Byun-re-01. The bacteria were pre-engineered to express 3D8 scFv. They exerted antiviral-activity upon challenge with MNV, in an *in vivo* setting. This looked promising for that particular purpose but as preservative candidate they were not able to show a high survival rate which hindered them to be further used as preservative agents [16]. We then attempted to select potential probiotic bacteria hence we screened more than 200 colonies from the ICR mice's intestines. Using 16S rDNA, most isolates were identified as L. *reuteri* strains with 99.47% similarity with those seen in L. *reuteri* JCM 1112. In the other hand, their gyrB DNA showed 99.64% similarity to L. *reuteri* strain I49. These two profiles indicated that even though they were phylogenetically related but they were certainly a new strain that differ from the two aforementioned stains (JCM 1112 and I49) (Figure 4). As a result, this new strain might carry different biological characteristics and activities. We, therefore, have registered it on the NCBI genome database with an accession number CP029613 as L. *reuteri* Byun-re-01 [37].

L. *reuteri* Byun-re-01, compared to *L. paracasei* ATCC 334, exerted much better survival in the gastrointestinal tract which is mainly characterized by its extremely low pH, thus abundant colonization was properly facilitated hence the strain was at a proper size to elicit the favorable effects. In another aspect, as both L. *reuteri* Byun-re-01 and *L. paracasei* ATCC 334 did not translocate to the livers, they did not induce deteriorating effects especially in livers as the detoxifying organ in the body. This is substantial as bacterial products might affect extra intestinal sites especially Kupffer cells, which are resident macrophages, in livers. These cells would respond by producing toxic forms of oxygens such as superoxide radicals, peroxide anion, single oxygen and hydroxyl radical, after D-galactoamine-induced endotoxin activation. As such, if bacteria migrated and compromised the portal circulation, Kupffer cells are accountable for this clearance. Disturbance of this activity might cause systemic effects of gut barrier failure or endotoxin might reach the systemic circulation which is holistically harmful [38]. Undamaged status of livers post-oral administration of both of L. *reuteri* Byun-re-01 and *L. paracasei* ATCC 334 were evident as seen in the level of serum AST/ALT which were all at normal range as did the control group.

We, next, questioned if the bacteria are safe for consumption because literally in every aspect one thing needs to get approval prior to utilization and in this specific case is their safety needs to have validation [39]. To address this, we measured body weights and food intake post-oral administration of L. *reuteri* Byun-re-01 and observed insignificant changes in body weight and daily food intake in L. *reuteri* Byun-re-01 –treated compared to that of control, suggesting that the bacteria did not cause appetite loss or maintain appetite in the same level as that of imperturbed/untreated control counterpart. We, unfortunately, did not go any further to draw probable causative connection. However, the disfavored events of type 2 diabetes mellitus (T2DM) might serve as a great example. This is principally a clinical manifestation of altered gut microbiota which is beneficially modified by probiotic treatment which is principally is aimed at maintaining glucose homeostasis which involves insulinotropic and satiety effects mediated by peptide tyrosine tyrosine (PYY) or glucagon-like peptide 1 (GLP1) which further weaken pro-inflammatory cytokines and enhance insulin sensitivity through upregulation of AMPK signalling. It is also acknowledged that interventions with probiotics displayed mixed findings thus far, implying more works for elaboration. Notwithstanding, as the dynamics of body weights as well as daily food intakes in L. *reuteri* Byun-re-01 –treated group are so close to that seen in control, we presumed that L. *reuteri* Byun-re-01 was safe for consumption until other future studies bring more solid outcome (Figure 7 and Table 3).

We, next, evaluated the advantage of L. *reuteri* Byun-re-01-treatment in protecting mice from MNV infection. We found that L. *reuteri* Byun-re-01 was capable of magnifying the expression of IFN- β and IFN-γ at mRNA level. Although this increase was also detected in *L. paracasei*-treated groups (strain ATCC 334 and KACC 11452) but L. *reuteri* Byun-re-01-treated group demonstrated double magnification than that of the *L. paracasei* groups. This loaded evidence that probiotic bacteria modulated local immune response by producing anti-inflammatory cytokines and inducing regulatory T cells (Tregs) in such complex pathways to assure local intestinal defense [34,40]. We, in this particular study, did not immune-phenotype the specific cells that were accountable for the cytokine productions since we stressed our work at discovery of novel probiotic bacteria to enrich our perspectives on their diversity which further elicited potential for commercial utilization. However, many reports have associated the anti-inflammatory cytokines with dendritic cells and T cells as the producers [40-42]. While Kawashima et al. depicted that DCs produced IFN-β after being challenged with Lactic Acid Bacteria (LAB) double-stranded RNA (dsRNA) which subsequently induced production of IFN-γ by T cells, Gutierrez-Merino et al. recently elucidated that the production of, specifically, IFN-I as a response to LAB necessitated intracellular sensors stimulators of IFN genes (STING) and Mitochondrial Antiviral Signaling (MAVS) to a lesser extent [41,42]. This IFN production was carried on by macrophages population [42]. All these are intriguing to investigate to generate loads of evidences to understand both how probiotic LAB activate their protective properties to defend physiological state of the host, in this specific case is the mice, and how this complex orchestration is hampered.

We next addressed subsequent query on how our L. *reuteri* Byun-re-01 elicited local protection in the intestine of the host against MNV, which in this study was represented by strain CR6. CR6 was

reported to be nonlethal strain that acquired stronger virulence by converting a nucleotide which resulted in amino acid substitution F514I in the viral capsid. This substitution led to beyond 10,000-fold higher replication in systemic organs [43]. To do so, we administered the bacteria to the mice *via* oral route daily for a couple of weeks. As comparison, we made two counterpart groups that received *L. paracasei* ATCC 334 and L. *reuteri* KACC 11452, separately. Stools were sampled at day 5-,7-,9- and 14- after the mice were infected with MNV CR6. Our result indicated that all groups that pre-treated with those three bacteria had suppressed frequencies of MNV viral capsid expression compared to that shown in MNV group which literally had no prior oral administration of bacteria. L. *reuteri* Byun-re-01 outperformed the other two groups with the reduction reached 6.6 folds lower at all sampling points (Figure 9). An interesting outlook from this results is also about L. *reuteri* Byun-re-01 had a stronger capability of hindering magnification of the viral capsid than their phylogenetically close counterpart L. *reuteri* KACC 11452, which was derived from human. Thereby we came into a thought that antiviral efficacy was not only determined by the biological properties of the respective bacteria but also by the origin of the bacteria. As seen in our data that L. *reuteri* Byun-re-01 had significantly obstructed the multiplication of the viral capsid of MNV CR6 compared to that seen in L. *reuteri* KACC 11452, a distinguished aspect between the two was literally their origins. L. *reuteri* Byun-re-01 was mice-derived and applied back to the mice host. This hinted that the bacteria had been educated by the microenvironment or undergone commensalism with other gut-niche microbiome in such a way that they are ready to encounter viral attacks that might skew the overall homeostasis of the digestive system.

While we discerned inhibitory activity against MNV *via* dynamics of the frequencies of viral capsid mRNA, Lange-Starke et al. previously in 2014 evaluated it from different perspective [44]. The team reported that a mixture of complex metabolite that present in the Cell-Free Supernatants (CFS) of L. curvatus was attributed to the inhibitory activity against MNV, which was further indicated to be a bacteriocin for its proteinaceous nature [44]. These two different approaches seem to be complementary one another and potentially generate new knowledge when are applied together.

Next step in our study was to identify the probiotic markers on a transcriptomic platform. This enabled us to detect some valuable profiles that were virulence-related factors be that, in descending order, dltA, dltD, sortase, hemolysin III, EBP and FbpA (fibronectin-binding-protein A). Specifically, we identified exopolysaccharides, important probiotic effector molecules involved in biofilm formation that facilitates the colonization of lactic acid bacteria in the intestines. L. *reuteri* Byun-re-01 also harbored general stress adaptation proteins, chaperone GroES, GroEL, DnaK, DnaJ, and F0F1 ATP synthase subunits. The level of expression of each gene was gauged as fragments per kilobase of transcript per million mapped reads. Our results demonstrated that the expression levels of most genes were high as illustrated in Figure 10. These suggested that L. *reuteri* Byun-re-01, derived from the original murine host, could more efficiently colonize in the intestines and persisted for a longer period in mice than that of bacteria originated from foreign organisms. In addition, this provided beneficial effects to the host.

Complete genome sequencing and transcriptome analysis of the newly isolated L. *reuteri* Byun-re-01 depicted the expression of several virulence and stress-related genes (Figure 10). The virulence factors included dltD (resistant to low pH conditions), dltA (ability to colonize the GIT), sortase, and hemolysin III, which are required for invasion and adherence to gut tissues [29]. Stress-related genes, including DnaK, DnaJ, GroEL, and GrpE, are involved in long-term acid stress resistance [45-47]. These four genes are heat-shock proteins in probiotics. The universal stress proteins required to overcome physiologic pressure and maintain the host–microbe interaction were highly expressed in L. *reuteri* Byun-re-01 [29]. The gene encoding exopolysaccharide biosynthesis protein, which is a probiotic effector molecule important for forming biofilm in the gut, was also present [29]. These *in silico* results support our experimental results and could be the basis for confirming that L. *reuteri* Byun-re-01 is safe for the host in future studies [48-50].

CONCLUSION

Overall, our study introduced novel probiotics isolated from mice, L. *reuteri* Byun-re-0, which was safe, confers an immunomodulatory effect, and thus was advantageous to control MNV infection in mice. This study also proposed a new framework to evaluate the safety of probiotics by combining *in vivo* and *in silico* experimental techniques. The combination would provide rapid and efficient assessment from *in silico* analysis and in-depth analysis through *in vivo* experiments.

DATA AVAILIBILITY

The complete genome of L. *reuteri* Byun-re-01 has been deposited at GenBank under accession number CP029613. The transcriptome data generated from Illumina sequencing were deposited in GenBank under accession number SRR8988597.

ETHICS STATEMENT

All animal procedures performed in this study were reviewed, approved, and supervised by the Institutional Animal Care and Use Committee of Sungkyunkwan University (SKKUIACUC2018-10-07-3).

AUTHOR CONTRIBUTIONS

DK, M-jC, E-JK, SL, and SJB designed the experiments and concepts. DK, M-jC, E-JK, and YL performed the experiments, analyses, and data interpretation. The manuscript was drafted by DK, IN, HGJS, and E-JK, HGJS. The study was supervised by SL. All authors discussed the results and commented on the manuscript.

FUNDING

This work was supported by a grant from the Next-Generation BioGreen 21 Program (Project No. PJ01328302), Rural Development Administration, Republic of Korea.

CONFLICT OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Wu QJ, Zheng XC, Wang T, Zhang TY. Effects of dietary supplementation with oridonin on the growth performance, relative organ weight, lymphocyte proliferation, and cytokine concentration in broiler chickens. BMC Vet Res. 2018;14(1):1-6.

2. Jose NM, Bunt CR, Hussain MA. Comparison of microbiological and probiotic characteristics of lactobacilli isolates from dairy food products and animal rumen contents. Microorganisms. 2015;3(2):198-212.

3. Yap MN. The double life of antibiotics. Mo Med. 2013 ;110(4):320-324.

4. Hughes P, Heritage J. Antibiotic growth-promoters. Feed Tech. 2002; 6:20–22.

5. Ishibashi N, Yamazaki S. Probiotics and safety. Am J Clin Nutr. 2001;73(2):465s-470s.

6. Kang JY, Lee DK, Ha NJ, Shin HS. Antiviral effects of *Lactobacillus* ruminis SPM0211 and Bifidobacterium longum SPM1205 and SPM1206 on rotavirus-infected Caco-2 cells and a neonatal mouse model. J Microbiol. 2015;53(11):796-803.

7. Oh NS, Joung JY, Lee JY, Kim Y. Probiotic and anti-inflammatory potential of *Lactobacillus* rhamnosus 4B15 and *Lactobacillus* gasseri 4M13 isolated from infant feces. PloS one. 2018;13(2):1-15.

8. Saxelin M, Chuang NH, Chassy B, Rautelin H, Mäkelä PH, Salminen S, et al. Lactobacilli and bacteremia in southern Finland, 1989–1992. Clin Infect Dis. 1996;22(3):564-566.

9. Kochan P, Chmielarczyk A, Szymaniak L, Brykczynski M, Galant K, Zych A, et al. *Lactobacillus* rhamnosus administration causes sepsis in a cardiosurgical patient—is the time right to revise probiotic safety guidelines?. Clin Microbiol Infect. 2011;17(10):1589-1592.

10. Borriello SP, Hammes WP, Holzapfel W, Marteau P, Schrezenmeir J, Vaara M, et al. Safety of probiotics that contain lactobacilli or bifidobacteria. Clin Infect Dis. 2003;36(6):775-780.

11. Arias A, Emmott E, Vashist S, Goodfellow I. Progress towards the prevention and treatment of norovirus infections. Future Microbiol. 2013;8(11):1475-1487.

12. Duizer E, Schwab KJ, Neill FH, Atmar RL, Koopmans MP, et al. Laboratory efforts to cultivate noroviruses. J Gen Virol. 2004;85(1):79-87.

13. Costantini VP, Whitaker T, Barclay L, Lee D, McBrayer TR, Schinazi RF, et al. Antiviral activity of nucleoside analogues against norovirus. Antivir Ther. 2012;17(6):981-991.

14. Lee JH, Bae SY, Oh M, Kim KH, Chung MS. Antiviral effects of mulberry (Morus alba) juice and its fractions on foodborne viral surrogates. Foodborne Pathog Dis. 2014;11(3):224-229.

15. Hoang PM, Cho S, Kim KE, Byun SJ, Lee TK, Lee S. Development of *Lactobacillus* paracasei harboring nucleic acid-hydrolyzing 3D8 scFv as a preventive probiotic against murine norovirus infection. Appl Microbiol Biotechnol. 2015;99(6):2793-2803.

16. Cho S, Kim D, Lee Y, Kil EJ, Cho MJ, Byun SJ, et al. Probiotic *Lactobacillus* paracasei expressing a nucleic acid-hydrolyzing minibody (3D8 ScFv) enhances probiotic activities in mice intestine as revealed by metagenomic analyses. Genes. 2018;9(6):276.

17. Yoon JH, Lee ST, Park YH. Inter-and intraspecific phylogenetic analysis of the genus Nocardioides and related taxa based on 16S rDNA sequences. Int J Syst Bacteriol. 1998;48(1):187-194.

18. Kim OS, Cho YJ, Lee K, Yoon SH, Kim M, Na H, et al. Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. Int J Syst Evol Microbiol. 2012;62(3):716-721.

19. Kim D, Cho MJ, Cho S, Lee Y, Byun SJ, Lee S. Complete Genome Sequence of *Lactobacillus reuteri* Byun-re-01, isolated from mouse small intestine. Microbiol Resour Announc. 2018;7(17):1-2.

20. Rhoads A, Au KF. PacBio sequencing and its applications. Genomics, Proteomics Bioinforma. 201;13(5):278-289.

21. Lee I, Kim YO, Park SC, Chun J. OrthoANI: an improved algorithm and software for calculating average nucleotide identity. Int J Syst Evol Microbiol. 2016;66(2):1100-1103.

22. McCartney SA, Thackray LB, Gitlin L, Gilfillan S, Virgin IV HW, Colonna M. MDA-5 recognition of a murine norovirus. PLoS Pathog. 2008;4(7):1-7.

23. Oozeer R, Furet JP, Goupil-Feuillerat N, Anba J, Mengaud J, Corthier G. Differential activities of four *Lactobacillus* casei promoters during bacterial transit through the gastrointestinal tracts of human-microbiota-associated mice. Appl Environ Microbiol. 2005;71(3):1356-1363.

24. Nguyen TD, Kang JH, Lee MS. Characterization of *Lactobacillus plantarum* PH04, a potential probiotic bacterium with cholesterol-lowering effects. Int J Food Microbiol. 2007;113(3):358-361.

25. González-Hernández MB, Perry JW, Wobus CE. Neutral red assay for murine norovirus replication and detection in a mouse. Bio-protocol. 2013;3(7).

26. Roy KR, Chanfreau GF. Robust mapping of polyadenylated and non-polyadenylated RNA 3′ ends at nucleotide resolution by 3′-end sequencing. Methods. 2020;176:4-13.

27. Lara-Villoslada F, Sierra S, Martín R, Delgado S, Rodríguez JM, Olivares M, et al. Safety assessment of two probiotic strains, *Lactobacillus* coryniformis CECT5711 and *Lactobacillus* gasseri CECT5714. J Appl Microbiol. 2007;103(1):175-184.

28. Hacker J, Kaper JB. Pathogenicity islands and the evolution of microbes. Annu Rev Microbiol. 2000;54(1):641-679.

29. Lebeer S, Vanderleyden J, De Keersmaecker SC. Genes and molecules of lactobacilli supporting probiotic action. Microbiol Mol Biol Rev. 2008;72(4):728-764.

30. Bennedsen M, Stuer-Lauridsen B, Danielsen M, Johansen E. Screening for antimicrobial resistance genes and virulence factors *via* genome sequencing. Appl Environ Microbiol. 2011;77(8):2785-2787.

31. Bourdichon F, Casaregola S, Farrokh C, Frisvad JC, Gerds ML, Hammes WP, et al. Food fermentations: microorganisms with technological beneficial use. Int J Food Microbiol. 2012;154(3):87-97.

32. Wei YX, Zhang ZY, Liu C, Malakar PK, Guo XK. Safety assessment of Bifidobacterium longum JDM301 based on complete genome sequences. World J Gastroenterol. 2012;18(5):479.

33. Senan S, Prajapati JB, Joshi CG. Feasibility of genome-wide screening for biosafety assessment of probiotics: a case study of *Lactobacillus* helveticus MTCC 5463. Probiotics Antimicrob Proteins. 2015;7(4):249-258.

34. Bui VT, Tseng HC, Kozlowska A, Maung PO, Kaur K, Topchyan P, et al. Augmented IFN-γ and TNF-α induced by probiotic bacteria in NK cells mediate differentiation of stem-like tumors leading to inhibition of tumor growth and reduction in inflammatory cytokine release; regulation by IL-10. Front Immunol. 2015;6:576.

35. Becquet P. EU assessment of enterococci as feed additives. Int J Food Microbiol.2003;88(2-3):247-254.

36. Yeo S, Lee S, Park H, Shin H, Holzapfel W, Huh CS. Development of putative probiotics as feed additives: validation in a porcine-specific gastrointestinal tract model. Appl Microbiol Biotechnol. 2016;100(23):10043-10054.

37. Kim YA, Keogh JB, Clifton PM. Probiotics, prebiotics, synbiotics and insulin sensitivity. Nutr Res Rev. 2018 ;31(1):35-51.

38. Osman N, Adawi D, Ahrne S, Jeppsson B, Molin G. Probiotic strains of *Lactobacillus* and Bifidobacterium affect the translocation and intestinal load of Enterobacteriaceae differently after D-galactosamine-induced liver injury in rats. Microb Ecol Health Dis. 2005;17(1):40-46.

39. Simon O, Jadamus A, Vahjen W. Probiotic feed additives-effectiveness and expected modes of action. J Anim Feed Sci. 2001;10:51-68.

40. Galdeano CM, Cazorla SI, Dumit JM, Vélez E, Perdigón G. Beneficial effects of probiotic consumption on the immune system. Ann Nutr Metab 2019;74(2):115-124.

41. Kawashima T, Ikari N, Watanabe Y, Kubota Y, Yoshio S, Kanto T, et al. Double-stranded RNA derived from lactic acid bacteria augments Th1 immunity *via* interferon-β from human dendritic cells. Front Immunol. 2018;9:27.

42. Gutierrez-Merino J, Isla B, Combes T, Martinez-Estrada F, Maluquer De Motes C. Beneficial bacteria activate type-I interferon production *via* the intracellular cytosolic sensors STING and MAVS. Gut Microbes. 2020;11(4):771-788.

43. Walker FC, Hassan E, Peterson ST, Rodgers R, Schriefer LA, Thompson CE, et al. Norovirus evolution in immunodeficient mice reveals potentiated pathogenicity *via* a single nucleotide change in the viral capsid. PLOS Pathog. 2021;17(3):e1009402.

44. Lange-Starke A, Petereit A, Truyen U, Braun PG, Fehlhaber K, Albert T. Antiviral potential of selected starter cultures, bacteriocins and D, L-lactic acid. Food Environ Virol. 2014;6(1):42-47.

45. Lim EM, Ehrlich SD, Maguin E. Identification of stress-inducible proteins in *Lactobacillus* delbrueckii subsp. bulgaricus. Electrophoresis. 2000;21(12):2557-2561.

46. Lorca GL, De Valdez GF, Ljungh A. Characterization of the protein-synthesis dependent adaptive acid tolerance response in *Lactobacillus* acidophilus. J Mol Microbiol Biotechnol. 2002;4(6):525-532. [Google Scholar]

47. Lee K, Lee HG, Pi K, Choi YJ. The effect of low pH on protein expression by the probiotic bacterium *Lactobacillus reuteri*. Proteomics. 2008;8(8):1624-1630.

48. Changotra H, Jia Y, Moore TN, Liu G, Kahan SM, Sosnovtsev SV, et al. Type I and type II interferons inhibit the translation of murine norovirus proteins. J Virol. 2009; 83(11):5683-5692.

49. Nice TJ, Baldridge MT, McCune BT, Norman JM, Lazear HM, Artyomov M, et al. Interferon-λ cures persistent murine norovirus infection in the absence of adaptive immunity. Science. 2015;347(6219):269-273.

50. Nice TJ, Robinson BA, Van Winkle JA. The role of interferon in persistent viral infection: insights from murine norovirus. Trends Microbiol. 2018;26(6):510-524.