In Vivo Characterization and Genome-based Approach to *Lactobacillus Johnsonii* Byun-jo-01 and Its Probiotic Properties

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Abstract

To develop new antiviral probiotics, bacteria were isolated from the microbiome in a murine intestine. In 16S rDNA sequence analysis, most isolates were identified as *Lactobacillus johnsonii*. These isolates were further assessed using whole-genome sequencing through the Illumina and PacBio platform, which revealed that the isolates were new strains. A novel probiotic strain, *Lactobacillus johnsonii* Byun-jo-01, was evaluated to determine its probiotic characteristics of safety, immune modulation, and antiviral efficacy against murine norovirus. Oral administration of *L. johnsonii* Byun-jo-01 was demonstrated to be safe in mice in terms of body weight, food intake, and bacterial translocation. Additionally, the expression levels of IFN-beta and IFN-gamma induced by *L. johnsonii* Byun-jo-01 in the small intestines of mice were higher than those in mice fed *L. paracasei* ATCC 334 and *L. reuteri* KACC 11452. Among the three different bacterial strains used in this study, *L. johnsonii* Byun-jo-01 showed the highest antiviral efficacy against murine norovirus, reducing the viral titer in fecal samples by 28 times compared with mice infected with murine norovirus. To support those *in vivo* experiments, genome-based data mining was performed to investigate which genes related to probiotic-specific markers were highly expressed in this isolate. Specifically, DnaK, GroEL, GroES, and GrpE, which are involved in the acid adaptation required to overcome the harsh *in vivo* condition, were highly expressed. Taken together, these results suggest that host-originated probiotics can be more effective than bacteria isolated from other sources, such as fermented food.

Introduction

Intestinal microbiota are involved in the host's overall immune system and have both beneficial and deleterious effects on health depending on the interactions between intestinal microorganisms and the host. Microbial diversity in the gut normally allows the gut to act as a bioreactor (Bäckhed et al. 2005). Within that healthy microbial diversity, the genus *Lactobacillus* is a probiotic that provides an immune boost to human beings and other animals when ingested in adequate amounts (Ishibashi and Yamazaki 2001). Despite the long clinical success of probiotic therapy, questions have been raised about its safety (Gasser 1994). Cases are very rare, but probiotic infections such as bacteremia and endocarditis mediated by *Lactobacilli* and *Bifidobacterium* in immunocompromised patients have been reported (Doron and Snyderman 2015; Gourié et al. 2012). Therefore, selecting probiotics through a rigorous screening process has been of great interest.

Noroviruses are the leading cause of viral gastroenteritis and are known worldwide to account for about 18 percent of diarrheal cases (de Graaf et al. 2016; Lopman et al. 2016). Outbreaks of human norovirus occur continuously worldwide, and infections cause vomiting and diarrhea in people of all ages (Fankhauser et al. 1998; Hansman et al. 2010; Hutson et al. 2004; Lopman et al. 2002). Despite the continued public-health emphasis on norovirus infection, effective vaccines targeting norovirus have not been developed. Because human norovirus is incompatible with cell culture systems, most research on norovirus has focused on murine norovirus, which shares characteristics with human norovirus (Kim et al. 2015; Wobus et al. 2006).

The absence of a laboratory analysis system for human norovirus has hindered the development of antiviral therapeutics. Only a specific part of the virus, virus-encoded cysteine protease (3CLpro), has been targeted; other research has sought methods to prevent infection (Rocha-Pereira et al. 2014). Interest in norovirus therapeutics that use probiotics or recombinant probiotics from the *Lactococcus* and *Lactobacillus* genera has been increasing (Aboubakr et al. 2014; Hoang et al. 2015).

We previously reported that recombinant *Lacticaseibacillus paracasei* ATCC 334 expressing the 3D8 scFv protein had antiviral effects against murine norovirus. When *L. paracasei* ATCC 334 expressing 3D8 scFv was orally administered to mice, the population of the intestinal probiotic *Pediococcus acidilactici* was increased in their intestines, whereas the population of *Helicobacter* species decreased (Cho et al. 2018).

Even though *L. paracasei* ATCC 334 had an antiviral effect, this *Lacticaseibacillus* isolated from cheese has a fairly short retention time in animals. Because the immune enhancement effect in the host is expected to increase with the duration in the intestine, lactic acid bacteria were isolated from a host microbiome to screen for probiotics that have a high survival rate and long intestinal duration in animals.

Many previous reports have used probiotics as antiviral agents against various viruses. Most of those studies have shown that lactic acid bacteria increase a host's innate immunity and induce resistance to the virus. When *Lactobacillus gasseri* SBT2055 was administered to mice, the survival rate of mice infected with the H1N1 PR8 strain was increased, and the expression of antiviral genes such as myxovirus resistance 1 (Mx1) and 2'-5' oligoadenylate synthetase 1A (Oas1a) in lung tissues was up-regulated (Nakayama et al. 2014). Various antiviral effects have been reported from *Lactobacillus*, but it is important to investigate the characteristics and antiviral mechanisms of newly isolated *Lactobacillus* strains because each *Lactobacillus* has a variety of antiviral mechanisms.

Advances in next-generation sequencing (NGS) techniques have resulted in easy availability of genomes of many *Lactobacillus* species (Dong et al. 2017; Inglin et al. 2017; Kazou et al. 2017; Kim et al. 2018). Safety assessment of probiotics has focused on searching for safe and/or harmful genes through *in silico* analysis (Wei et al. 2012; Zhang et al. 2012).

Our purpose in this research is to isolate *Lactobacillus* strains from a mouse microbiome and find antiviral probiotics which have strong colonization ability in mouse intestine. We used an *in vivo* system to assess *L. johnsonii* Byun-jo-01, a strain isolated from the small intestines of mice, to determine its antiviral efficacy, immune-boosting effect, and safety. We also performed transcriptome sequencing to supplement the *in vivo* data and screen for safety genes through data mining.

Materials And Methods

Sample collection and isolation of bacteria

We aseptically sampled the small intestines of specific pathogen-free ICR mice (DBL, Republic of Korea). After sampling, the extracted tissues were divided into three parts (duodenum, jejunum, ileum) and

homogenized with stainless steel beads (NEXT ADVANCE, USA) and phosphate-buffered saline (PBS). To isolate *Lactobacillus* species, each part of the intestine was spread on MRS (Man-Rogosa-Sharpe) plates that were then incubated at 37°C for 48 hours. Each colony was selected from the plates and cultured on MRS medium. Using microscopy, bacteria with a rod shape were selected and maintained in MRS medium supplemented with 80% glycerol at -80°C. Another probiotic strain, *Lacticaseibacillus paracasei* ATCC 334, was provided by Dr. Jos Seegers (Falcobio, Netherlands), and *Limosilactobacillus reuteri* KACC 11452 and *Lactobacillus johnsonii* KCCM 32825, which were isolated from humans, were provided by the Rural Development Administration (Republic of Korea).

Primer design

All the primer sets used in this study are listed in **Table 1**. Previously described by Yoon *et al* (Kim *et al.* 2012), the 9F and 1542R primers, which target bacterial 16S rDNA as a universal primer, were used to identify the *Lactobacillus* species. The reference sequences of cytokines interferon (IFN)- β and IFN- γ were retrieved from GenBank (www.ncbi.nlm.nih.gov) with the following accession numbers (NM_010510.1, NM_008337.3, NM_031168.1, and NM_013693.2, respectively). Additionally, the primer set for murine norovirus detection was obtained from the sequence of the MNV capsid protein (JQ237823.1). All primers were designed using the Primer 3 program.

DNA isolation

Lactobacillus genomic DNA was prepared for bacterial identification and whole-genome sequencing. First, 200 μ l of bacterial seed on 4.8ml of MRS medium was used for DNA preparation. When the OD₆₀₀ value reached 1.0, ampicillin (0.1mg/ml) was administered for 1 hour at 37°C to weaken the cell wall of the *Lactobacillus*. Bacterial cells were collected by centrifugation at 13,000 $\times$ g for 1 min and washed twice with PBS. Then the bacterial pellet was treated with a lysis mixture (20 mg/ml lysozyme, 50 U/ml Mutanolysin in Tris-EDTA buffer) for 1 hour at 37°C. Genomic DNA was isolated using a G-spin™ Genomic DNA Extraction Kit (for Bacteria) (iNtRON Biotechnology, Republic of Korea) according to the manufacturer's protocols. The concentration of genomic DNA was measured at 260nm with a spectrophotometer.

Amplification of 16S rRNA

The prepared genomic DNA was used to identify the isolated *Lactobacillus* by amplifying the 16S rRNA gene. Amplification of the 16S rRNA amplicon was performed with the 9F and 1542R universal bacterial primers using a thermal cycler (Bio-Rad T100™). The PCR conditions were as follows: initial denaturation of DNA for 5 min at 95°C, followed by 35 cycles of denaturation of DNA for 30 sec at 95°C, an annealing step for 30 sec at 55°C, an extension step for 1 min 30 sec at 72°C, and final extension for 10 min at 72°C. PCR products were detected by electrophoresis with a 1% agarose gel stained with ethidium bromide, and target bands were visualized on a UV illuminator.

Identification of *Lactobacillus* species

The PCR product was purified from the gel extraction using a Zymoclean™ Gel DNA Recovery Kit (ZYMO RESEARCH, USA) according to the manufacturer's protocols. The 24-hour ligation step was performed using the pGEM®-T easy vector system (Promega, USA) at 16°C. The ligation mixture was transformed to a DH5α competent cell. To select the target genes, we used the blue-white screening procedure and cultured only white colonies in Luria-Bertani broth (Difco Laboratories, USA) supplemented with 100 µg/ml of ampicillin. Plasmid preparation was performed using a AccuPrep® Nano-Plus Plasmid Extraction Kit (BIONEER, Republic of Korea) according to the manufacturer instructions. The target gene was confirmed using restriction enzyme digestion (*Eco* RI), followed by Sanger/Capillary sequencing (Macrogen, Republic of Korea). Identification of the bacterial species was carried out using the EzTaxon-e database.

Phylogenetic analysis

The conserved 16S rRNA sequences of various *Lactobacillus johnsonii* reference strains and other species (*L. rhamnosus* GG, *L. fermentum* IFO 3956, and *L. paracasei* ATCC 334) were obtained from GenBank, including mouse isolates, to determine phylogenetic relationships. Before creation of a phylogenetic tree, we performed multiple alignments using the MEGA program (version 7). Phylogenetic relationships were identified through the Neighbor-Joining method with 1000 bootstrap replications.

Whole-genome sequencing, assembly, annotation

Whole-genome sequencing of *L. johnsonii* Byun-jo-01 was performed as previously reported (Kim et al. 2018). In brief, lysis agents were used (50U/ml Mutanolysin, 20mg/ml lysozyme) to weaken the bacterial cell wall before isolating the DNA, and then genomic DNA was prepared using a G-spin™ Genomic DNA Extraction Kit (for Bacteria) (iNtRON Biotechnology, Republic of Korea) according to the manufacturer's protocols. Using a combination of PacBio RS II (Pacific Biosciences, USA) and Illumina Hiseq2000 (Illumina, Inc., USA), whole genome sequencing was performed at Macrogen (Seoul, Republic of Korea), resulting in a complete genome. The assembly used to construct the complete genome was a two-step process. First, with only the raw sequencing data generated from PacBio sequencing, *de novo* assembly was carried out using SMRT Analysis software version 2.3.0 (Zhang et al. 2000). Second, to decrease the error rate in the genome assembly and improve genome completeness, hybrid assembly using Pilon (v1.21) (Walker et al. 2014) was conducted with raw data generated from Illumina sequencing. Putative coding genes were annotated with Prodigal version 2.6 software (Hyatt et al. 2010). Using both ARAGON version 1.2 and RNAmmer version 1.2 software (Lagesen et al. 2007), tRNA and rRNA genes were identified. The default parameters were used in all software. The complete genome sequence of *L. johnsonii* Byun-jo-01 was deposited in GenBank under accession number CP029614.

Comparison of circular genome

Using whole genome information from similar *L. johnsonii* strains uploaded in the NCBI genome database (<http://www.ncbi.nlm.nih.gov/genome>), genome completeness was confirmed. Seven *L. johnsonii* strains were obtained based on phylogenetic relationships: *L. johnsonii* strains NCC533, N6.2,

BS15, DPC60206, FI9785, UMNJ21, and UMNJ22, along with *L. johnsonii* Byun-jo-01. These strains were subjected to comparative analysis to identify similarities among them. Comparison of whole genomes was conducted using the BLAST Ring Image Generator (BRIG) (Alikhan et al. 2011).

Mice, virus, and cell culture

Depending on the purpose of the experiment, we used various kinds of mice. To isolate *Lactobacillus*, we used six-week-old, female, specific pathogen-free ICR mice (DBL, Republic of Korea). Female MNV-seronegative C57BL/6 mice (6 weeks) were used to test the antiviral efficacy of *Lactobacillus* isolates against MNV-1.CR6. All mice were acclimatized for 1 week before experiments and were housed under standard laboratory conditions. 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). RAW264.7 cells were maintained in Dulbecco's modified Eagle's medium (Hyclone, USA) containing 10% heat-inactivated fetal bovine serum (Alphabio, USA), 100 units/ml of penicillin, and 100 µg/ml of streptomycin at 37°C in a 5% CO₂ incubator. Murine norovirus GV/CR6/2005/USA (MNV) was provided from Washington University by Herbert W. Virgin and grown in RAW264.7 cells (McCartney et al. 2008).

Determination of survival and retention of *L. johnsonii* Byun-jo-01

To determine the survival and retention time of *L. johnsonii* Byun-jo-01 in the mouse gastrointestinal tract, we modified the pSLP111.3 vector (originally provided by Dr. Jos Seegers) by replacing the xylose-inducible promoter with a lactate dehydrogenase (LDH) constitutive promoter as previously described (Oozeer et al. 2005). Transformation of the pSLP111.3 vector into *L. paracasei* ATCC 334 and *L. johnsonii* Byun-jo-01 was carried out using the electroporation method as previously described (Hoang et al. 2015). Each transformed *Lactobacillus* was used to differentiate the administered *Lactobacillus* from the other commensal bacteria in the feces of mice. The mice were assigned to four experimental groups (three mice per group): 1) presence or absence of antibiotic pretreatment before the experiment and 2) the number of feedings. To maintain the pSLP111.3 vector and remove partially commensal bacteria from the small intestine, all mice were given water containing 3 µg/ml chloramphenicol for 24 h. Food and water were withdrawn for 18 h before the feeding trials. The mice were fed 10⁸ CFU of *L. paracasei* ATCC 334 or *L. johnsonii* Byun-jo-01 once or 3 times every 2 days using feeding needles (20 gauge). In the experimental group that did not receive antibiotics, *Lactobacillus* was administered directly without pretreatment. Fecal samples were collected from each mouse from day 1 to day 11 following the first administration of *Lactobacillus*. At each time point, the fecal samples were weighed and then homogenized in PBS, followed by serial dilution. Diluted fecal samples were inoculated on MRS plates containing 3 µg/ml of chloramphenicol. After anaerobic incubation at 37°C for 2 days, the viable counts in the fecal samples were determined, and the number of colony forming units (CFUs) of *Lactobacillus* per gram of feces was calculated. Negative control mice were fed only PBS.

Bacterial translocation

Translocation events, in which administered bacteria move from gut tissues to other organs, were confirmed as previously described (Nguyen et al. 2007). The liver and kidney were harvested aseptically, and each organ was homogenized in PBS using stainless steel beads (NEXT ADVANCE, USA). About 100 µl of homogenates and 20 µl of blood were inoculated on MRS agar and BHI (Brain Heart Infusion) agar (BD Biosciences, USA), and the MRS plates were incubated for 48 hours at 37°C. After 48 hours, incidence of translocation was expressed as number of mice in which bacteria were detected / number of total mice. We defined occurrence of an event as one or more colonies on the agar plates.

Immune-boosting mediated by *L. johnsonii* Byun-jo-01

Total RNA was prepared from small intestine tissues using TRI reagent (Molecular Research Center, Inc., USA). RNA concentrations were measured at 260nm absorbance using a spectrophotometer. cDNA was synthesized from 5 µg of total RNA using Moloney murine leukemia virus reverse transcriptase (Bioneer, Republic of Korea) according to the manufacturer's protocols. The following cytokines were quantitatively measured to compare expression levels induced by *Lactobacillus*: IFN-β and IFN-γ. Quantitative real-time PCR was conducted using SYBR Premix Ex Taq (TaKaRa, Japan) and a Rotor-Gene Q system (Qiagen, Australia). Data were analyzed using Rotor-Gene Q series software version 2.3.1 (Qiagen, Australia).

Antiviral efficacy against MNV CR6

In addition to the *L. paracasei* ATCC 334 strain, the antiviral activity test was performed using *L. johnsonii* KCCM 32825, which originated from a human, as a control probiotic. After administration of 10⁸ CFU of *Lactobacillus* (*L. paracasei* ATCC 334, *L. johnsonii* KCCM 32825, or *L. johnsonii* Byun-jo-01) to the mice, 10⁵ PFU of MNV was given orally. Feces were collected on days 5, 7, 9, and 14 after viral infection. Fecal RNA was extracted using a Quick-RNATM Fecal / Soil Microbe Microprep kit (ZYMORESEARCH, USA). cDNA was synthesized from 5 µg of total RNA using CellScript cDNA Synthesis Master Mix (CellSafe, Republic of Korea) with a random primer. The viral titer was analyzed among experimental groups using quantitative real-time PCR.

Transcriptome sequencing

L. johnsonii Byun-jo-01 grown in pH4.8 conditions to mimic its niche was used for RNA preparation with a NucleoSpin® RNA kit (MACHEREY-NAGEL, Germany). To prevent DNA contamination in the total RNA samples, DNase was added, and then the purified mRNA was isolated using Ribo-Zero rRNA Removal Kit (Bacteria) (Illumina, USA), completely removing the rRNA from the samples. The cDNA library was constructed with a TruSeq RNA Sample Prep Kit v2 for library preparation according to the manufacturer's instructions. Paired-end libraries (200–400bp insert sizes) were sequenced using the HiSeq2000 platform (Macrogen, Republic of Korea).

Mapping and Fragments Per Kilobase of transcript per Million (FPKM) calculation

The raw data (4 fastq files) generated from transcriptome sequencing were deposited in GenBank under accession number SRR9001782. Adaptor sequences were removed by Trimmomatic 0.32 software, and then only the trimmed reads were mapped onto the reference sequence (the genome of *L. johnsonii* Byun-jo-01) using BMap (short-read aligner) (Bushnell 2014). Default parameters were used for all software during this process. Probiotic-related markers were searched using data mining and previously published papers.

Statistical analysis

All analyses were carried out in triplicate and assessed using GraphPad Prism (GraphPad software version 5). Data are presented as means $\pm$ SEM. $P < 0.05$ was considered significant. One-way analysis of variance (ANOVA) was used for statistical analysis.

Results

Identification of *Lactobacillus* isolates from mouse gastrointestinal tract

The divided tissues (duodenum, jejunum, ileum) of mice were used to find probiotic candidates. MRS agar plates were used to specifically isolate *Lactobacillus* from intestinal tissues. Viable bacterial colonies were selected for identification using universal 16S rRNA primers as previously described (Kim et al. 2012). Most isolates were found to be *L. johnsonii*, except for a few species (*Lactobacillus intestinalis*, putative *Lactocaseibacillus paracasei*), and this isolate showed 100% similarity with various *L. johnsonii* strains including *L. johnsonii* N6.2 and *L. johnsonii* DPC 6026. The *L. johnsonii* isolates finally selected for further experiments were isolated in the jejunum, the part of the small intestine between the duodenum and ileum.

Phylogenetic relationships based on 16S rRNA of *L. johnsonii* strains

To determine phylogenetic relationships between the isolated strain and previously reported similar strains, a phylogenetic tree was created using 16S rDNA sequences, which are known to be conserved in microorganisms. The sequence data for the reference strains, *L. fermentum*, *L. brevis*, *L. plantarum*, *L. salivarius*, *L. paracasei*, and *L. crispatus*, and various *L. johnsonii* stains were retrieved from the NCBI database (www.ncbi.nlm.nih.gov). Sequence alignment using 16S rRNA (average 1500bp) was carried out using the CLUSTAL X program, followed by construction of a phylogenetic tree based on the Neighbor-Joining method with 1000 bootstrap replicates. The result, as shown in **Fig. 1**, indicates that the *L. johnsonii* mouse isolates belong to same clade as various other *L. johnsonii* strains.

Genomic characterization of *L. johnsonii* Byun-jo-01

The 16S rRNA gene is a standard for bacterial identification because of its presence in all bacteria. However, it has been suggested that basing the identification of bacterial strains on only the 16S rRNA gene is limiting (Stackebrandt 2006). To further identify this isolate, therefore, we conducted whole-genome sequencing using both the Illumina Hiseq 2000 (2 x 100 bp paired-end sequencing; Illumina, Inc.,

USA) and PacBio RS II (Pacific Bioscience, USA) platforms. General genome features and a circular map of the chromosome of *L. johnsonii* Byun-jo-01 are shown in **Table 2** and **Fig. 2**, respectively. According to the genome features table, single-molecule real-time (SMRT) cell sequencing with 20-kb inserts yielded 1,847,047,342 bp (182,640-bp-long reads). Draft genome assembly was carried out using 182,640 reads generated from PacBio sequencing. To improve the accuracy of genome assembly, a total of 81,648,222 short reads generated from the Illumina sequencing was used for hybrid assembly. That two-step assembly process generated a complete genome with a size of 1,959,519 bp (N50 values 1,959,519) and a GC content of 34.7%. The annotation process indicated that the genome of *L. johnsonii* Byun-jo-01 consists of 1,781 coding sequences, 21 rRNAs, and 77 tRNAs. As shown in **Fig. 3**, the distribution of clusters of orthologous groups (COG) in *L. johnsonii* Byun-jo-01 was identified. Among the COG functional categories, J (translation, ribosomal structure, and biogenesis), G (carbohydrate transport and metabolism), M (cell wall/membrane/envelope biogenesis), and K (translation) were the most abundant in the genome.

In addition to the 16S rRNA sequences, bacterial identification was carried out on a whole-genome level, and *L. johnsonii* Byun-jo-01 was found to be a novel strain. For identification, similar *L. johnsonii* strains (*L. johnsonii* NCC 533, *L. johnsonii* strain BS15, *L. johnsonii* N6.2, *L. johnsonii* strain UMNLJ22, *L. johnsonii* strain UMNLJ21, and *L. johnsonii* DPC6026) were retrieved from the NCBI genome database. To visualize the similarity in the whole genomes of the *L. johnsonii* strains, BRIG was used. The overall similarity of the genomes is shown in **Fig. 4**. Using BLAST, the overall similarity of the *L. johnsonii* genome with the reference strain was numerically compared and found to be greater than 98% (**Table 3**).

Survival and retention of *L. johnsonii* Byun-jo-01 in the gastrointestinal tract

The ability of *L. johnsonii* Byun-jo-01 to reach and survive in the gastrointestinal tract of mice was evaluated by counting viable colonies of *L. johnsonii* Byun-jo-01 in fecal samples after oral administration under various antibiotic treatments and numbers of feeding trials. To distinguish between administered bacteria and other intestinal bacteria in the fecal samples, *L. paracasei* ATCC 334 and *L. johnsonii* Byun-jo-01 were transformed using the pSLP111.3 expression vector containing a chloramphenicol-resistant gene and LDH constitutive promoter (Hoang et al. 2015), and those transformed bacteria were used as the selection tool instead of wild type *Lactobacillus*. For all the *Lactobacillus* used in the experiment, 10^8 CFUs were administered via oral administration. The survivability of *L. johnsonii* Byun-jo-01 over time after administration is shown in **Fig. 5**. To facilitate placement of the bacteria in the intestine, commensal bacteria were removed by giving the mice water containing chloramphenicol. As expected, all administered bacteria were detected in the feces under any experimental condition. In the mice fed antibiotics, *L. johnsonii* Byun-jo-01 was present in the feces 24h after administration, reaching a level of 1×10^6 CFU/g, and that level decreased gradually over time. Unlike *L. paracasei* ATCC 334, *L. johnsonii* Byun-jo-01 survived for about 11 days after both single and triple doses (**Figs. 5a and 5b**). However, without antibiotics, the number of CFUs 24h after administration was relatively small, and the duration of *L. johnsonii* Byun-jo-01 was much shorter than in the antibiotics

condition (**Figs. 5c and 5d**). The difference between these results indicates that the antibiotics allowed *L. johnsonii* Byun-jo-01 to establish and remain in the intestine for a long time.

Safety assessment of *L. johnsonii* Byun-jo-01

Mice were given *L. johnsonii* Byun-jo-01 at a dosage of 10^8 CFU / mouse daily for 2 weeks to discern any safety issues. After administration of *Lactobacillus*, changes in body weight, food intake, and organ weight were determined. Prior to the experiment, all subjects started at the same body weight. During the experiment, no abnormal behavior was observed in any experimental group compared with the control mice. The body weight increased slightly in all experimental groups over time (**Fig. 6**), but none of the changes were statistically significant. Additionally, no detectable changes in food intake were observed, as shown in Table 3. Food intake was measured every 2 days because daily intake varied widely among individuals. The weight and appearance of the liver, small intestine, and spleen were confirmed after sacrifice. There was no significant change in organ weight between the *Lactobacillus*-treated mice and the negative control mice, and no significant lesions were found (**Fig. 7**). Taken together, these findings suggest that the safety of *L. johnsonii* Byun-jo-01 did not differ significantly from that of control *L. paracasei* ATCC 334 when used as a probiotic.

Analysis of bacterial translocation

Even though the safety test found no abnormalities, as described above, the bacterial translocation test is necessary before a strain can be developed and used as a probiotic. Bacterial translocation can occur when the balance of the intestinal microbiome is disrupted and the junction of intestinal epithelial cells is weakened by any bacteria. This allows bacteria present in the intestine to migrate from the gastrointestinal tract to sites such as the spleen and bloodstream (Berg 1995). We judged translocation to have occurred if even a single colony was found on the MRS or BHI agar plates. In both the negative control mice and the *L. johnsonii* Byun-jo-01-fed mice, no events were detected at any site, as shown in **Table 4**. Only one event occurred in the liver of a mouse treated with *L. paracasei* ATCC 334. There were no significant differences in translocation incidence between the control mice and the *L. johnsonii* Byun-jo-01-fed mice. Translocated bacteria in the liver can cause damage and increase the levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Tian et al. 2015). Although we found no bacterial colonies on the plates from the organs, we performed an additional analysis to determine whether the serum ALT and AST levels were within the normal ranges. All tested mice had AST and ALT levels within the normal ranges (AST: 54–298 U/L, ALT: 17–77 U/L). The AST level in mouse serum was 110 U/L in control mice, 105 U/L in *L. paracasei* ATCC 334–treated mice, and 109 U/L in *L. johnsonii* Byun-jo-01–treated mice. The ALT level was 38, 44, and 38 (U/L), respectively. Taken together, these results imply that *L. johnsonii* Byun-jo-01 isolated from murine intestines has no safety issues as a probiotic in mice.

Immune-boosting activity of *L. johnsonii* Byun-jo-01

Intestinal microbiota form an immunological barrier in the host and play an important role in maintaining the intestinal immune system through interactions among themselves and between intestinal microorganisms and intestinal immune cells (Delcenserie et al. 2008). *Lactobacillus*, as reported in many references in which lactic acid bacteria were applied to animal models for clinical trials, are particularly known as health-promoting factors (Galdeano and Perdigon 2006b; Gill et al. 2000; Hori et al. 2002). *Lactobacillus* strains regulate host immunity and induce secretion of various cytokines in different ways, depending on the type of *Lactobacillus* (Delcenserie et al. 2008). To determine how *L. johnsonii* Byun-jo-01 enhances intestinal immunity, we evaluated cytokines: IFN- β and IFN- γ . To determine how expression of the selected cytokines was increased by the administered strains (*L. paracasei* ATCC 334, *L. reuteri* KACC 11452, and *L. johnsonii* Byun-jo-01), a dose of 10^8 CFU of *Lactobacillus* was administered to mice daily for 2 weeks, as in the safety assessment, and the cytokine profiles in intestinal tissue were examined using quantitative real-time PCR. We expected that the lactic acid bacteria isolated from the host microbiome would confer a greater immune enhancement on the original host, so we used human-derived *L. reuteri* KACC 11452 as another control group. Compared with the negative control mice, as shown in **Fig. 8**, the relative mRNA expression of IFN- β and IFN- γ in mice treated with *L. johnsonii* Byun-jo-01 was about 9.04-fold and 23-fold higher, respectively. The expression levels of IFN- β and IFN- γ mediated by *L. johnsonii* Byun-jo-01 were 3.82 and 6.39 times higher, respectively, than those mediated by *L. paracasei* ATCC 334.

Antiviral activity test of *L. johnsonii* Byun-jo-01 against murine norovirus

The C57BL/6 mouse model was chosen for this experiment because it is particularly feasible for murine norovirus infection (Tomov et al. 2013). The most important factor required to control murine norovirus infection is innate immunity, involving proteins such as STAT-1, which is a primary mediator of both type I and type II interferon responses (Platanias 2005; Wobus et al. 2006). As illustrated in **Fig. 8**, *L. johnsonii* Byun-jo-01 increased the expression of IFN- β and IFN- γ , thus conferring anti-viral efficacy to mice. To confirm whether *Lactobacillus* isolates protected MNV-infected mice, oral gavage was performed for 2 weeks prior to virus infection. Because the MNV CR6 strain remains chronically *in vivo*, no viral capsid mRNA was detected in the stool until 3 days after infection (data not shown). Using feces collected on days 5, 7, 9, and 14 after viral infection, we analyzed the antiviral effects of the *Lactobacillus* isolates over time (**Fig. 9**). On the 7th day after infection, viral capsid mRNA was maximally expressed; compared with negative control mice on day 7, the number of viral genes in the feces of mice that received *L. johnsonii* Byun-jo-01 decreased more than 80-fold. Additionally, *L. johnsonii* KCCM 32825 isolated from humans was used to compare its efficacy with that of the murine strains isolated from the host microbiome. *L. johnsonii* Byun-jo-01 induced the highest anti-viral state in mice, 13 times better than that from the *L. johnsonii* human isolates. The most striking finding of this experiment was that the efficacy of the isolated *Lactobacillus* was far superior to that of *Lactobacillus* species isolated from other sources, such as fermented foods.

Characterization of probiotic-related markers using transcriptome sequencing

To investigate the probiotic-related genes expressed in its original niches, *L. johnsonii* Byun-jo-01 was cultured in MRS media at pH4.8, which mimics intestinal pH conditions, and total RNA was extracted. RNA, with the DNA completely removed, was used to purify mRNA, followed by transcriptome sequencing using the Hiseq2000 platform. Total reads (26,701,034 bp and 29,291,286 bp) were generated, and adaptor sequences were removed using Trimmomatic 0.32 software in the default condition for exact mapping. Only processed reads were used for the mapping procedure through BMap, followed by screening of the probiotic-related genes to support our *in vivo* safety data. On the basis of published literature (Lebeer et al. 2008; Papadimitriou et al. 2015), we searched and analyzed common probiotic-related markers to determine their expression levels. In the definition of probiotics, virulence factors, a probiotic factor and an adaptation factor, are essential to the host–microbe interaction (Lebeer et al. 2008). The genes involved in the stress response, DnaK, DnaJ, GroES, GrpE, and the F0F1 ATP synthase subunits, were discovered, and the FPKM value of those transcripts was greater than 20,000 (**Fig. 10**). Additionally, genome analysis revealed many adhesion-related factors, such as sortase, dltD (D-adenylation of LTA), hemolysin III, and fibronectin-binding protein, which are responsible for adhesion to the extracellular matrix of epithelial cells (Azcarate-Peril et al. 2008). The most characterized probiotic marker, exopolysaccharide, was detected, but its expression level was much lower than that of the other markers. Overall, the expression levels of the markers involved in the stress response were up-regulated upon exposure to bile acid or gastric juice and were significantly higher than the markers involved in adhesion.

Discussion

Lactobacillus compose much of the intestinal diversity in domestic animals and are widely used as probiotics. Probiotics, commonly known to be safe, have been used in a variety of pharmaceutical drugs as well as dairy products after numerous clinical trials (Salminen et al. 1998). Nonetheless, rare cases of infection by *Lactobacillus* and *Bifidobacterium* have been reported, such as bacteremia and endocarditis in immunocompromised patients (Salminen et al. 1998). Therefore, in developing new probiotic treatments, probiotics must first be scientifically verified and undergo safety evaluations, rather than being applied directly to clinical trials (Borriello et al. 2003).

Development of NGS techniques has allowed easy access to genomic information about many microbes. Therefore, it is unnecessary to prove the safety of each probiotic experimentally in *in vitro* and *in vivo* systems. The databases can demonstrate the safety of a probiotic by enabling researchers to explore probiotic-specific genes and safety-related genes through data mining, which is much cheaper and more efficient than experimental methods (Senan et al. 2015). Thus, we attempted to prove the safety of a probiotic by combining *in vivo* and *in silico* systems in a thorough screening and thereby provide a new framework for safety evaluation.

Based on the criteria for probiotic selection, we identified isolated *Lactobacillus* at the genus, species, and strain levels using 16S rDNA sequencing and whole-genome sequencing through NGS technology. By

comparing similarities with strains reported previously, we found that our isolate was a novel strain, *L. johnsonii* Byun-jo-01 (**Figs. 1–2, Tables 2–3**).

Oral toxicity was evaluated by administering a high dose of the potential probiotic strain *L. johnsonii* Byun-jo-01 to mice for 2 weeks. As a result, we found no adverse effects to body weight or food intakes compared to normal mice, indicating that the potential probiotic did not show oral toxicity or have any detrimental effect on the health of mice (**Table 4, Figs. 6–7**). These results suggest that administration of this potential probiotic has no deleterious effects (Lara-Villoslada et al. 2009).

Indigenous bacteria can move from the gut to other tissues in an opportunistic infection, which produces toxicity markers and can cause side effects (Steffen and Berg 1983). No bacterial translocation of the potential probiotics tested here was observed in mice, except in one case treated with *L. paracasei* ATCC 334 (**Table 5**). In terms of safety assessment, the evaluation factors described above differed little from those of normal mice, confirming that administration of the selected *Lactobacillus* had no effect on the health of mice.

A survival-rate experiment was conducted to determine whether the mouse strain administered could reach the intestine despite exposure to gastric acid or bile acid, and the results showed a longer retention time than the control probiotic strain in all experimental conditions (**Fig. 5**). In particular, when given along with antibiotics, the Byun-jo-01 strain survived in the gastrointestinal tract much longer than the ATCC 334 strain, indicating that microbial reduction by antibiotics treatment helped the administered *Lactobacillus* colonize the gut (Hill et al. 2010). In the literature, the retention time of *Lactobacillus* isolated from other sources was relatively short (Su et al. 2007), whereas the mouse strain in this study originated from a mouse microbiome and was superior in the original host (mice).

Lactobacilli can lead to both innate and adaptive immunity by binding in specific patterns, microbe-associated molecular patterns (MAMPs), with pattern recognition receptors in immune cells (Abreu 2010; Kawai and Akira 2010; Wells et al. 2010). Induction of cytokines and chemokines after this binding can vary according to the combination of MAMPs and Toll-like receptors (Abreu 2010; Kawai and Akira 2010). According to many researchers, probiotics such as *L. casei*, *L. rhamnosus*, and *L. plantarum* can elicit different immunomodulatory effects in a strain-specific manner (Vitini et al. 2000). *In vivo* studies demonstrated that *L. casei*, *L. rhamnosus*, and *L. plantarum* affect both systemic and mucosal immunity by stimulating immune cells in the intestine and binding antigen-presenting cells, followed by expression of inflammatory (TNF- α , IFN- γ , IL-12) and regulatory cytokines (IL-4, IL-10) (Galdeano and Perdigon 2006a). Although we confirmed that intestinal immunity was increased by the Byun-jo-01 strain, especially by an increase in cytokines associated with antiviral defense such as IFN- β and IFN- γ (**Fig. 8**), the mechanism by which this strain affects the gut-associated immune system should be further elucidated.

To analyze the antiviral efficacy of the enhanced immunity, we verified the effects of the Byun-jo-01 strain using an MNV-infected mouse model. Because MNV strain CR6 is not acute but has enteric persistence (Nice et al. 2015), we demonstrated the preventive effect of the Byun-jo-01 strain against MNV from the

3rd day post-infection. Compared with the control mice (MNV-infection only), the lowest level of virus titer was found in mice treated with the Byun-jo-01 strain over the entire period, and the Byun-jo-01 strain was more effective than *L. johnsonii* KCCM 32825. Our findings in this study suggest that the benefits derived from the original host produced anti-viral efficacy because the safety and effective immune enhancement of the Byun-jo-01 strain were better than those of any other probiotics tested.

To characterize the potential probiotics validated in the *in vivo* system and to support the results of the preceding experiments, we conducted both whole-genome sequencing and transcriptome sequencing analysis to detect probiotic-specific genes. The two main categories used to describe probiotics are called adaptation factors and probiotic factors; in combination, they are described as virulence factors (Lebeer et al. 2008). We detected many virulence factors, such as dltD, sortase, and hemolysin III, involved in adherence in the gut. Among them, dltD was most highly expressed in the Byun-jo-01 strain (**Fig. 10**). Additionally, stress-related genes involved in acid adaptation (long-term acid stress resistance), DnaK, DnaJ, GroEL, GroES, and F0F1 ATP synthase subunits, were highly expressed. According to previous research, two specific proteins of *L. johnsonii* NCC 533, elongation factor Tu (EF-Tu) and the heat shock protein GroEL, are adhesive factors (Bergonzelli et al. 2006; Granato et al. 2004). Our *in silico* findings confirm expression of genes that can play a role as potential probiotics. These results are supportive of our *in vivo* findings.

Taken together, our results can be used as a foundation for the basic process of more thorough screening of potential probiotics.

Declarations

Funding

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Conflicts of interest

The authors declare no competing interests.

Availability of data and material

The complete genome of *L. johnsonii* Byun-jo-01 has been deposited at GenBank under the accession number CP029614. The transcriptome data generated from Illumina sequencing were deposited in Genbank under the following accession number (SRR9001782).

Author's contributions

D.K., M.-J.C., E.-J.K., S.L., S.J.B. designed the experiment and concepts. D.K., M.-J.C. performed the experiments and analyzed the data with E.-J.K. Manuscript was drafted by D.K., E.-J.K., S.L.

D.K., M.-J.C., E.-J.K., S.L. performed analyses and interpretation of data. All authors discussed the results and commented on the manuscript.

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Tables

Table 1. Primers used in this study

Gene	Forward (5'→3')	Reverse (5'→3')	Reference
16S rDNA	GAGTTTGATCCTGGCTCAG	AGAAAGGAGGTGATCCAGCC	(Yoon et al. 1998)
GAPDH	TGGCAAAGTGGAGATTGTTGCC	AAGATGGTGATGGGCTTCCCG	NM_002046
IFN-beta	TTACACTGCCTTTGCCATCCAA	TCCCACGTCAATCTTTCCTCTT	NM_010510.1
IFN-gamma	ACTGGCAAAGGATCGTGAC	GACCTGTGGGTTGTTGACCT	NM_008337.3
MNV capsid protein	CTCTCAGCCATGTACACCGG	TAGGGTGGTACAAGGGCAACAA	JQ237823.1

Table 2. General genome information for *L. johnsonii* Byun-jo-01

<i>L. johnsonii</i> Byun-jo-01	
Sequencing platforms	PacBio RS II / Illumina Hiseq2000
Assembler	PacBio SMRT Analysis 2.3.0 / Pilon (v1.21)
Number of reads	182,640 (PacBio) / 81,648,222 (Illumina)
Genome coverage	635
Genome size (bp)	1,959,519
G+C content (%)	34.7
Predicted CDS	1781
Number of contigs	1
Number of rRNA genes	21
Number of tRNA genes	77
N50 (bp)	1,959,519

Table 3. Comparison of whole-genome similarity between *L. johnsonii* Byun-jo-01 and reference strains

Strain	Coverage	Similarity	Origin	Accession
NCC 533	93%	99%	Homo sapiens	AE017198
BS15	90%	99%	Homo sapiens	CP016400
N6.2	90%	99%	Rattus	CP006811
UMNLJ22	80%	98%	Meleagris gallopavo	CP021704
UMNLJ21	80%	98%	Meleagris gallopavo	CP021703
DPC 6026	90%	98%	Sus scrofa domesticus	CP002464

Table 4. Measurement of food intake after administration of *Lactobacillus*

Strain	Food intake (g)
Negative control	7.88 ± 0.65
<i>L. paracasei</i> ATCC 334	7.27 ± 1.04
<i>L. johnsonii</i> Byun-jo-01	7.28 ± 0.87

Values are mean ± SD for n=3

Table 5. Bacterial translocation from gastrointestinal tract to other tissues

	Bacteria	Blood	Kidney	Liver
MRS agar	Negative control	0/3	0/3	0/3
	<i>L. paracasei</i> ATCC 334	0/3	0/3	1/3
	<i>L. johnsonii</i> Byun-jo-01	0/3	0/3	0/3
BHI agar	Negative control	0/3	0/3	0/3
	<i>L. paracasei</i> ATCC 334	0/3	0/3	0/3
	<i>L. johnsonii</i> Byun-jo-01	0/3	0/3	0/3

The value indicates the number of animals with translocation / total mice

Figures

Figure 1

Phylogenetic relationships of *L. johnsonii* mouse isolates based on 16S rRNA sequences with reference sequences. Construction of the phylogenetic tree was performed using the Neighbor-Joining method. An average of 1500 nucleotides of 16S rRNA was aligned, and bootstrap values (1000 replicates) are

indicated at the branch points. All values were greater than 40%. *Limosilactobacillus*, *Levilactobacillus*, *Lactiplantibacillus*, *Ligilactobacillus*, *Lacticaseibacillus*, and *Lactobacillus* species used in this result were as follows: various *L. johnsonii* strains including *L. johnsonii* mouse isolate, *L. fermentum*, *L. crispatus*, *L. brevis*, *L. paracasei*, *L. salivarius*, *L. plantarum*, *L. rhamnosus*, *L. iners*, *L. jensenii*, *L. acidophilus*, *L. delbrueckii*, and *L. helveticus*. The scale represents 0.1 nucleotide substitutions per position.

Figure 2

Circular representation of the *L. johnsonii* Byun-jo-01 genome. Representation of the chromosome map of *Lactobacillus johnsonii* Byun-jo-01. Marked characteristics are displayed from the 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 visualized using circos.

Figure 3

Clusters of orthologous group (COG) functional categories in *L. johnsonii* Byun-jo-01. Distribution of COG functional categories in *L. johnsonii* Byun-jo-01. Putative 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

Similarity of the whole-genome sequence of *L. johnsonii* Byun-jo-01 with those of other *L. johnsonii* species. Comparison of the whole genome of *L. johnsonii* Byun-jo-01 was performed, and representation of the aligned circular map is shown using the BLAST Ring Image Generator (BRIG) with the default setting. To compare similarity, the genomes of *L. johnsonii* NCC533, *L. johnsonii* N6.2, *L. johnsonii* BS15, *L. johnsonii* DPC60206, *L. johnsonii* FI9785, *L. johnsonii* UMNJ21, and *L. johnsonii* UMNJ22 were obtained from the NCBI genome database (<http://www.ncbi.nlm.nih.gov/genome>).

Figure 5

Persistence of *L. johnsonii* Byun-jo-01 in the gastrointestinal tract under various conditions. Under antibiotic treatment conditions, 108 CFUs of *Lactobacillus* strains (*L. paracasei* ATCC 334 and *L. johnsonii* Byun-jo-01) were administered once (a) and three times every two days (b). To analyze congenital persistence (the ability of *L. johnsonii* Byun-jo-01 to colonize the gut tissue in the absence of antibiotics), the *Lactobacillus* strains were administered once (c) and three times every two days (d). Data are presented as the mean $\pm$ SEM. (●: Negative control, ■: *L. paracasei* ATCC 334, ▲: *L. johnsonii* Byun-jo-01)

Figure 6

Comparison of weight change in mice treated with Lactobacillus and Lacticaseibacillus strains. *L. paracasei* ATCC 334 and *L. johnsonii* Byun-jo-01 were administered to mice daily for 2 weeks. During the administration period, the body weight of the mice was measured daily. Data are presented as the mean $\pm$ SEM of n=3.

Figure 7

Comparison of organ weights among mice treated with Lactobacillus and Lacticaseibacillus strains. Mice treated with *L. paracasei* ATCC 334 as a positive probiotic were compared with mice treated with *L. johnsonii* Byun-jo-01. After 2 weeks, the mice were sacrificed, and their livers, spleens, and small intestines were harvested to measure weight. Data are presented as the mean $\pm$ SEM of n=3.

Figure 8

Immune boosting activity of *L. johnsonii* Byun-jo-01 in gut tissues. The expression levels of cytokines induced by *Lactobacillus* strains in gut tissues were measured using qRT-PCR. An additional control probiotic strain, *L. reuteri* KACC 11452 (originating from humans), was used to compare the immune-boosting activity of *L. johnsonii* Byun-jo-01. The relative mRNA expression of cytokines (a. IFN- β , b. IL-6, c. IFN- γ , d. TNF- α) was quantified. GAPDH was used as an internal control, and the relative mRNA expression levels of the cytokines induced by *Lactobacillus* strains were calculated using the delta delta CT method. Data are presented as the mean $\pm$ SEM of $n=3$. * $p<0.05$, ** $p<0.01$

Figure 9

Antiviral efficacy of *L. johnsonii* Byun-jo-01 against murine norovirus strain CR6. To analyze the anti-MNV effect of *Lactobacillus* strains, they were administered to MNV-infected mice. Fecal samples collected on days 5, 7, 9, and 14 after infection were used for analysis. *L. johnsonii* KCCM32825 was used as another control probiotic. The relative expression of MNV capsid protein in fecal samples was analyzed. GAPDH was used as an internal control, and the relative mRNA level of capsid protein was calculated using the delta delta CT method. Data are presented as the mean $\pm$ SEM of $n=3$. ** $p<0.01$, *** $p<0.001$

Figure 10

Screening of probiotic-specific markers through transcriptome data. To find probiotic-specific markers, a transcriptome analysis was conducted to identify various adaptation factors and probiotic factors. The expression level for each gene is indicated using the FPKM (Fragment Per Kilobase of transcript per Million mapped reads). Data are presented as the mean $\pm$ SEM of $n=2$.