In vivo and *in silico* characterization of *Lactobacillus reuteri* SKKU-OGDONS-01, a potential probiotic from chicken intestine

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Research

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Abstract

Background: *Lactobacillus reuteri* SKKU-OGDONS-01 was isolated from chicken intestines for further development as an antiviral feed additive. This study aimed to investigate probiotic properties of chicken isolates in mice model and *in silico* analysis.

Results: Compared to known probiotics, *Lactobacillus paracasei* ATCC 334, *Lactobacillus reuteri* SKKU-OGDONS-01 showed immune-boosting effects despite short persistence in the mice intestine. Especially, the expression levels of IFN-β and IFN-γ were increased 4 and 40 times higher than those of the control mice. In proportion to the immune-boosting effects elicited by chicken isolates, the antiviral efficacy against murine norovirus (MNV) was also remarkable. For the purpose of evaluating the potential for development as feed additives, the expression levels of probiotic markers such as long-term acid adaptation, stress response, and adhesion-related proteins were investigated using *in silico* method, and the results showed that these proteins were expressed at high levels in chicken isolate.

Conclusion: Our study demonstrated that chicken isolate, *Lactobacillus reuteri* SKKU-OGDONS-01 can also elicit high probiotic properties in mice even though it originated in chicken. We expect that this chicken isolate will be able to induce much higher probiotic activity in chickens to develop feed additives for poultry.

Keywords: *Lactobacillus reuteri* SKKU-OGDONS-01, probiotics, antiviral efficacy, probiotic marker, cytokine

Background

Rapid development of mass breeding of livestock has caused extreme stress in animals and has created a system very vulnerable to infectious diseases [1]. Eventually, mass breeding will adversely affect livestock, leading to decreases in production. To prevent infectious diseases, antibiotics have been included in livestock feed, but governments have been banning use of antibiotics because of concerning about antibiotic resistance and transfer of antibiotic-resistant genes [2]. The poultry industry is therefore working to develop feed additives, such as probiotics and chlorella that can replace antibiotics and still increase the immunity of livestock to infectious diseases [3–5].

Lactobacillus is the dominant bacterial genus with probiotic characteristics in the avian alimentary tract [6, 7]. In order for newly isolated lactic acid bacteria to be developed as pharmaceutical agents such as antiviral feed additives, their function as probiotics must be verified under strict scientific approaches and bacteria should be chosen from the microbial flora in the gastrointestinal tract of human or other animals on the basis of criteria [3].

In our previous study, we found that, after administration of transgenic Lactobacillus paracasei ATCC 334 expressing the 3D8 scFv (single chain variable fragment) protein to mice, the population of Helicobacter species in the intestine decreased, and the population of Pediococcus acidilactici (a probiotic) increased [8]. In addition, transgenic L. paracasei ATCC 334 showed antiviral efficacy against murine norovirus

(MNV) [9]. However, the cheese-derived Lactobacillus is expected to have relatively short persistence and survival in the intestine, which can weaken its probiotic effects. Therefore, the development of new probiotics with better efficacy from its own intestinal samples should be essential. We performed this study to find probiotic candidates from microflora of chicken intestines and evaluate probiotic properties using mouse and in silico systems before applying to chickens.

Results

Identification of Lactobacillus species from chicken intestines

To identify potential probiotics, tissue from the small intestine of a chicken was extracted. After homogenizing the extracted tissue, MRS agar plates were used to selectively isolate gram-positive bacteria such as Lactobacillus, Bifidobacteria, and Enterococcus. Among the many colonies, the first screening process used microscopy to select rod-shaped bacteria. For further analysis, the 16S rRNA gene was used for bacterial identification. In the 16S rRNA sequencing analysis, most of the bacteria isolated from the small intestine were identified as Lactobacillus reuteri. Based on the result from EZcloud™ (chunlab, https://www.ezbiocloud.net), the 16S rRNA sequences were 99.60% similar to that of the L. reuteri JCM 1112 strain. Total 200 isolates including Lactobacillus intestinalis and Lactobacillus crispatus, from the intestine samples were isolated, but because most bacteria in the gastrointestinal tract were L. reuteri, we selected those isolates for further characterization.

Construction of a phylogenetic tree based on the 16S rRNA genes of L. reuteri strains

The phylogenetic relationships among the L. reuteri isolates and similar bacterial species were determined by comparing 16S rRNA sequences, which are known to be conserved in microorganisms. The reference sequences of the other Lactobacillus species (various L. reuteri strains (L. reuteri ZLR003, L. reuteri I5007, L. reuteri ATCC 53608, L. reuteri I49, L. reuteri JCM 1112, L. reuteri DSM 20016, L. reuteri IRT, L. reuteri TD1), L. fermentum IFO 3956, L. brevis ATCC 367, L. plantarum WCFS1, L. salivarius UCC 118, L. rhamnosus GG, L. paracasei ATCC 334, L. iners DSM 13335, L. johnsonii NCC 533, L. jensenii JV-V16, L. delbrueckii subsp. Bulgaricus ATCC 11842, L. crispatus ST1, L. helveticus CNRZ32, and L. acidophilus NCFM) were retrieved from the NCBI database (www.ncbi.nlm.nih.gov). Alignment of 16S rRNA sequences (average length, 1500 bp) was conducted using the CLUSTAL X program, and then we determined the phylogenetic relationships among bacterial species. To reconfirm the similarity identified in the EZcloud™ database, a phylogenetic tree was constructed using the Neighbor-Joining method with 10000 bootstrap replications, and we found that the L. reuteri chicken isolates belong to the same clade as similar L. reuteri strains (Fig. 1).

Characterization of a novel strain using high-throughput sequencing

Although 16S rRNA sequence analysis is widely used for routine identification of microorganisms, some researchers have suggested that additional experiments be conducted for further identification, even when a 16S rRNA sequence is 99% similar to an existing bacterial strain [10]. To determine whether the

Lactobacillus strain isolated from a chicken was a new strain, we performed high-throughput sequencing using both the Illumina Hiseq 2000 (2 × 100 bp paired-end sequencing) and PacBio RS II platforms. Table 2 and Fig. 2 provide information about the genomic features and a circular map of L. reuteri SKKU-OGDONS-01 drawn by CIRCOS (http://circos.ca), respectively. The two-step assembly process was conducted using a total of 57,137,834 paired-end short reads generated from Illumina sequencing and 151,868 long reads generated by PacBio sequencing to make a complete genome. As the first step, draft genome assembly was conducted using the long reads, and the complete genome was finished using the short reads in the second step. The complete genome of L. reuteri SKKU-OGDONS-01 was 2,259,968 bp (N50 values 2,259,968) with a G + C content of 38.9%. Based on the PGAP result, the chromosome contained 2,165 genes, 1,941 putative coding sequences (CDS), 70 tRNA genes, and 18 rRNA genes. As shown in Table 3, we identified clusters of orthologous groups (COGs) in the genome of L. reuteri SKKU-OGDONS-01. The COG functional categories contain 2,165 genes, and the most abundant categories were J (translation, ribosomal structure, and biogenesis, 8.72%), X (mobilome: prophages, transposons, 8.68%), G (carbohydrate transport and metabolism, 5.66%), and E (amino acid transport and metabolism, 5.99%). Category S (function unknown, 6.57%) was also abundant.

Table 1
Specific primers used in this study

Gene	Forward (5'→3')	Reverse (5'→3')	Reference
16S rRNA	GAGTTTGATCCTGGCTCAG	AGAAAGGAGGTGATCCAGCC	[50]
GAPDH	TGGCAAAGTGGAGATTGTTGCC	AAGATGGTGATGGGCTTCCCG	NM_002046
IFN-β	TTACACTGCCTTTGCCATCCAA	TCCCACGTCAATCTTTCCTCTT	NM_010510.1
IFN-γ	ACTGGCAAAAGGATCGTGAC	GACCTGTGGGTTGTTGACCT	NM_008337.3
IL-6	AGTTGCCTTCTTGGGACTGA	TCCACGATTTCCCAGAGAAC	NM_031168.1
TNF-α	CGTCAGCCGATTTGCTATCT	CGGACTCCGCAAAGTCTAAG	NM_013693.2
MNV capsid protein	CTCTCAGCCATGTACACCGG	TAGGGTGGTACAAGGGCAACAA	JQ237823.1

Table 2

General genome information for L. reuteri SKKU-OGDONS-01

	L. reuteri SKKU-OGDONS-01
Sequencing platforms	PacBio RS II / Illumina Hiseq2000
Assembler	PacBio SMRT Analysis 2.3.0 / Pilon (v1.21)
Number of reads	151,868 (PacBio) / 57,137,834 (Illumina)
Genome coverage	451
Genome size (bp)	2,259,968
G + C content (%)	38.9
Number of genes	2,165
Predicted CDS	1,941
Number of contigs	1
Number of rRNA genes	18
Number of tRNA genes	70
N50 (bp)	2,259,968

Table 3

Identified clusters of orthologous groups (COGs) in L. reuteri SKKU-OGDONS-01

COG	Description	Count	Ratio (%)
J	Translation, ribosomal structure, and biogenesis	182	8.72
A	RNA processing and modification	0	0.00
K	Transcription	111	5.32
L	Replication, recombination, and repair	107	5.13
B	Chromatin structure and dynamics	0	0.00
D	Cell cycle control and cell division	35	1.68
Y	Nuclear structure	0	0.00
V	Defense mechanisms	51	2.44
T	Signal transduction mechanisms	51	2.44
M	Cell wall/membrane/envelope biogenesis	103	4.94
N	Cell motility	5	0.24
Z	Cytoskeleton	0	0.00
W	Extracellular structures	0	0.00
U	Intracellular trafficking and secretion	13	0.62
O	Posttranslational modification and chaperones	59	2.83
X	Mobilome: prophages and transposons	181	8.68
C	Energy production and conversion	60	2.88
G	Carbohydrate transport and metabolism	118	5.66
E	Amino acid transport and metabolism	125	5.99
F	Nucleotide transport and metabolism	77	3.69
H	Coenzyme transport and metabolism	65	3.12
I	Lipid transport and metabolism	51	2.44
P	Inorganic ion transport and metabolism	63	3.02
Q	Secondary metabolite biosynthesis and transport	22	1.05
R	General function prediction only	119	5.70
S	Function unknown	137	6.57

COG	Description	Count	Ratio (%)
Multi	Multiple COG category	169	8.10
Nohit	No hits against COG database	182	8.72
Total		2086	100

To determine whether the Lactobacillus isolated from the chicken was a novel strain, we conducted a comparative analysis based on the whole genome. The similarities among Lactobacillus strains were analyzed based on an OrthoANI algorithm, and a heatmap was generated to indicate similarities based on Average Nucleotide Identity (ANI) value (Fig. 3). As a result, L. reuteri SKKU-OGDONS-01 showed an average of 95% similarity to 9 different L. reuteri strains. Therefore, we registered the chicken-originated L. reuteri SKKU-OGDONS-01 in the NCBI genome database as a new strain. Information about this novel strain is accessible through accession number CP029615. Information about the sequencing reads (Sequence Read Archive number SRP162209) can be accessed through BioProject number PRJNA473291 and BioSample number SAMN09270376.

Survival rate of Lactobacillus strains in the gastrointestinal tract

We measured the survival rate and retention time in the small intestine, critical criteria of probiotics, to evaluate whether our potential probiotic strain can resist the strong pH of gastric and bile acids in the in vivo environment. To differentiate between commensal bacteria in the intestinal tract and the administered Lactobacillus, the probiotics were used in a transgenic form. To construct the transformed strains, we modified the pSLP111.3 vector by replacing the xylose-inducible promoter with an LDH (lactose dehydrogenase) constitutive promoter [9]. Then we used the chloramphenicol resistant gene to measure the survival rate and retention time of the administered Lactobacillus, as shown in Fig. 4. Antibiotics were treated prior to the administration of Lactobacillus so that the administered bacteria could easily settle into the intestine. The results showed no significant difference in survival or persistence compared with the L. paracasei ATCC 334 strain used as a control probiotic for either one or three administrations of Lactobacillus (Fig. 5a and 5b). However, we did find more colonies on the MRS plates after administration of L. reuteri SKKU-OGDONS-01 compared with administration of L. paracasei ATCC 334. When antibiotics were not given (to evaluate the congenital colonization activity of the chicken-originated strain), L. reuteri SKKU-OGDONS-01 did not survive for longer than the control Lactobacillus, just as we found with antibiotics (Fig. 5c and 5d). When L. reuteri SKKU-OGDONS-01 was administered three times every two days, colonies were observed at a relatively high level the day after administration (Fig. 5d). However, the chicken-derived Lactobacillus in mice had no noticeable advantage over the cheese-derived control in survival rate or retention time.

Safety aspects of L. reuteri SKKU-OGDONS-01

To assess the safety of L. reuteri SKKU-OGDONS-01, we administered a wild-type strain for 2 weeks instead of the transformed strain used in previous experiments, and then we measured and evaluated

health state, weight change, food intake, and organ weights of the mice. All the mice were same weight at the start of the experiment, and the weight among the experimental groups did not differ during administration. Likewise, the total amount of food intake was essentially the same among groups (Fig. 6a and 6b). Even in the results of phenotypic changes and organ weights (liver, small intestine, and spleen) after completion of Lactobacillus administration, no differences among groups were observed, as shown in Table 4. Thus, the health of the mice was unaffected by administration of L. reuteri SKKU-OGDONS-01.

Table 4. Organ weights of mice fed *Lactobacillus* strains

Values are mean ± SEM for n=3

Bacteria	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	0.62 ± 0.05	0.11 ± 0.02
L. reuteri SKKU-OGDONS-01	1.38 ± 0.11	0.73 ± 0.07	0.11 ± 0.01

Assessment of bacterial translocation

Before a Lactobacillus strain can be used as a probiotic, a thorough safety evaluation is needed, including a bacterial translocation test. We observed the alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, looking for elevations that occur when the liver is damaged by bacterial translocation. Bacterial translocation was defined as even a single colony of Lactobacillus in the liver, kidney, or blood of the subjects and assayed among experimental groups as shown in Table 5. Except for one incidence, no obvious translocation events (migration of bacteria from gut tissues to other organs and blood) were detected. Elevated AST and ALT levels, as toxicity indicators of translocation, were analyzed. The normal ranges for AST and ALT in mice are 54–298 U/L, and 17–77 U/L, respectively [11]. We found that the ALT value in the mice that received L. reuteri SKKU-OGDONS-01 was 27 ± 2.94, and the AST value was 71 ± 9.2, as shown in Table 6. Considering ALT and AST levels of the negative control mice were 33.6 ± 3.68 and 79.6 ± 22.3, respectively, L. reuteri SKKU-OGDONS-01 caused no liver damage. Furthermore, the ALT/AST levels in mice fed L. paracasei ATCC 334 were also in the normal range (ALT: 32.3 ± 8.33, AST: 91.3 ± 11.44). Therefore, this potential probiotic could be used safely for further purposes.

Table 5
Incidence of bacterial translocation

Agar	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 SKKU-OGDONS-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 SKKU-OGDONS-01	0/3	0/3	0/3
The value indicates the number of animals with translocation / total mice.				

Table 6. Serum analysis of mice treated with *Lactobacillus* strains

Values are mean ± SEM for n=3

Group	ALT (U/L)	AST (U/L)
Negative control	33.6 ± 3.68	79.6 ± 22.3
L. paracasei ATCC 334	32.3 ± 8.33	91.3 ± 11.44
L. reuteri SKKU-OGDONS-01	27 ± 2.94	71 ± 9.2

Immune-boosting effect of L. reuteri SKKU-OGDONS-01

Intestinal microbiota play a key role in forming a biofilm in the intestine of the host, blocking various substances coming from the outside and contributing to homeostasis of the immune system, especially by interacting with immune cells in the intestine [12]. In particular, Lactobacillus are known to be good probiotics, with beneficial health effects demonstrated in many clinical trials and previously accumulated data. Depending on the mode of action, probiotics have various ways to enhance immunity in the host [13–15]. Even though L. reuteri SKKU-OGDONS-01 did not show a long duration in the small intestines of mice, we compared the expression of the representative cytokines known to be elevated by probiotics such as interferon(IFN)- β, IFN-γ, tumor necrosis factor-alpha (TNF-α), and interleukin-6 [13, 16] through administration of different Lactobacillus strains (L. paracasei ATCC 334, L. reuteri KACC 11452, and L. reuteri SKKU-OGDONS-01, respectively) for two weeks at a dose of 10^8 CFUs daily. The expression of cytokines was compared using quantitative real-time PCR. As shown in Fig. 7, the relative expression of IFN-β and IFN-γ, known antiviral cytokines, increased by 4 and 40 times, respectively, in mice fed L. reuteri

SKKU-OGDONS-01 compared with mice without any treatment. We also confirmed that the expression of IFN-β and IFN-γ differed by 3 and 1.7 times, respectively, compared with mice treated with L. paracasei ATCC 334. The relative expression of TNF-α and interleukin-6 was 1.4 and 8.4 times higher, respectively, in mice fed L. reuteri SKKU-OGDONS-01 than in mice without any treatment. Our findings show that antiviral cytokines and IL-6, which act in opposing directions, increased at the same time, which differs from what was previously understood: a single Lactobacillus strain can induce expression of both pro- and anti-inflammatory cytokines.

Antiviral efficacy of L. reuteri SKKU-OGDONS-01 against murine norovirus

STAT-1 deficient ($STAT1^{-/-}$) mice are highly susceptible to MNV-1 infection [17, 18], but in wild-type mice, the virus has a relatively low infectivity. Among wild-type mice, C57BL/6 mice were reported to be suitable for studying MNV, so this experiment was conducted using those mice [19]. The most important factor in controlling MNV infection is innate immunity, such as STAT-1, which is a primary mediator of both type I and type II interferon responses [20, 21]. It has also been reported that IFN-γ plays a particularly crucial role [22]. As shown in Fig. 8, the expression of IFN-β and IFN-γ in the intestines of mice treated with L. reuteri SKKU-OGDONS-01 increased significantly, so we expected that the mice would experience antiviral effects against MNV in the same conditions. To test that expectation, 3 wild-type Lactobacillus strains, including L. reuteri SKKU-OGDONS-01, were administered to mice daily for 2 weeks before MNV infection, and Lactobacillus administration continued after infection. Due to the unique characteristics of the MNV CR6 strain, the viral capsid protein was not found in the feces at the early stage of infection; however, the viral protein began to be detected 5 days after infection (Fig. 8). Because the virus can replicate in vivo for a long time, fecal samples were collected on days 5, 7, 9 and 14 post-infection for analysis. The antiviral effect of the Lactobacillus strains was most remarkable on day 7 after infection. Compared with the mice that received only the virus, the amount of viral protein in mice treated with L. reuteri SKKU-OGDONS-01 decreased by more than 35 times. In the comparison with the human-originated L. reuteri KACC 11452, we found that the amount of viral protein differed by 10 times on the 7th day after infection. As with the tendency in the results of the immune-boosting experiments, the induction of antiviral cytokines was highest in mice fed L. reuteri SKKU-OGDONS-01. Thus, our novel strain had the best antiviral efficacy among the Lactobacillus strains tested.

Screening for probiotic-related markers using in silico analysis

Although we fully investigated the safety of L. reuteri SKKU-OGDONS-01 using our in vivo system, we explored probiotic-related markers using an in silico system to further support our results. For the in silico data mining analysis, we used the Illumina Hiseq2000 platform to obtain transcriptome information for L. reuteri SKKU-OGDONS-01 cultured in a pH 7.0 environment which mimic its original niche. The transcriptome information obtained through high-throughput sequencing is shown in Table 7. Total reads (2,688,348,749 and 2,501,571,444 bp) were generated, and only the reads from which the adapter sequences were removed were mapped to chromosome sequences through BBMap. Several genes defined as probiotic markers in the literature [23, 24] were found, and we investigated their expression

levels using FPKM values to determine how many of those genes were expressed by L. reuteri SKKU-OGDONS-01. When describing probiotics, the virulence factor is a combination of probiotic factor and adaptation factor, which are an essential part of microbe host by overcoming the strong pH of the stomach and the bile acid it encounters until it reaches the intestine [23]. Genes associated with the stress response and long-term acid adaptation, DnaK, DnaJ, GroES, GrpEL, GrpE, and the F0F1 ATP synthase subunits, were found in L. reuteri SKKU-OGDONS-01 (Fig. 9). When the expression levels of those genes were indicated in terms of FPKM, their average value was greater than 10,000. In addition, our data mining found sortase, dltD (D-adenylation of LTA), hemolysin III, and fibronectin-binding protein, which are all adhesion factors that allow the bacteria to adhere to the extracellular matrix of epithelial cells in the intestine [25]. The most well-known probiotic marker, exopolysaccharide, was also found, but it had a lower expression level than the other markers. Overall, several probiotic markers are highly expressed within L. reuteri SKKU-OGDONS-01, and these in silico data support our safety assessment of Lactobacillus in mice.

Table 7
Transcriptome data of L. reuteri SKKU-OGDONS-01

Index	LR-C-3	LR-C-4
Total base reads (bp)	2,688,348,749	2,501,571,444
Total reads (bp)	26,682,928	24,812,502
No. of processed reads (bp)	13,341,464	12,406,251
No. of mapped reads (%)	9,599,295 (71.95%)	10,977,992 (88.49%)
No. of failed-to-align reads (%)	783,882 (5.88%)	720,124 (5.80%)

Discussion

The productivity of chickens, which has developed rapidly during the past few decades, has brought great benefits to the poultry industry. However, as the number of chickens raised per unit area has continued to increase, the risk of serious damage from a disease outbreak has also increased [26]. Additionally, as chickens become more vulnerable to harmful bacteria, such as those in the genera E. coli, Salmonella, and Campylobacter in the intestines, the use of antibiotics to control them is increasing [27]. Therefore, to reduce the poultry industry's dependence on antibiotics, recent studies have focused on validating the efficacy of immune-boosters or probiotics in feed to induce immune enhancement.

The genus Lactobacillus dominates the bacterial population in the duodenal loops, small intestines, and ceca of chickens [28]. Because various kinds of Lactobacillus live in the digestive tract, their potential for use and development as probiotics is virtually infinite. The safety of probiotics in pharmaceutical drugs and dairy products has been verified in numerous clinical trials, but nonetheless very rare cases of infection have occurred, such as bacteremia and endocarditis by Lactobacillus and Bifidobacterium in

immunocompromised patients [29]. Therefore, development of new probiotics requires strict safety verification using scientific approaches [30].

The development of genome databases and high-throughput sequencing technology has provided easy access to microbial genome information for data mining, such as screening for probiotic markers [31, 32]. Therefore, it is now possible to investigate the safety of probiotics cheaply and efficiently using genome information rather than conducting expensive experiments [33]. Our purpose in this study was to test the safety of a newly selected Lactobacillus using a combination of in vivo and in silico systems and thereby suggest a new verification method.

In accordance with the reported selection criteria for probiotics, we first identified the genus, species, and strain of Lactobacillus using 16S rRNA and high-throughput sequencing. The 16S rRNA and whole-genome sequencing results showed that chicken-derived Lactobacillus were highly similar to previously reported Lactobacillus reuteri strains, but we did find a novel strain called L. reuteri SKKU-OGDONS-01 (Fig. 1–3, Table 2).

Oral toxicity after administration of Lactobacillus is one of the categories to be assessed in any safety evaluation. This potential probiotic strain, L. reuteri SKKU-OGDONS-01, did not have a detrimental effect on the weight, food intake, or health conditions of mice after 2 weeks of oral administration (Fig. 5, Table S3) [34].

Bacterial translocation, in which bacteria move from the intestine to other organs and blood, is a toxicity marker that can explain the side effects of probiotics [35]. No case of bacterial translocation occurred in mice treated with L. reuteri SKKU-OGDONS-01 (Table 5). The safety of this strain was thus experimentally verified in our safety assessment.

Persistence and survival in the intestinal tract, which are representative characteristics of probiotics, were analyzed under various experimental conditions. Our novel Lactobacillus strain isolated from chicken reached the intestines of mice, and we observed no differences in survival or persistence from the L. paracasei ATCC 334 used as a control probiotic in any experimental condition (Fig. 5). Previous reports have suggested that the survival rate of probiotics isolated from other substances could be relatively short [36], so our results with L. reuteri SKKU-OGDONS-01 were not unexpected. However, we administered L. reuteri SKKU-OGDONS-01 for a very short time, so its retention time should be further studied using long-term administration.

Lactobacillus can act as a bridge between innate and adaptive immunity because of its unique molecular pattern, called a microbe-associated molecular pattern (MAMP). Cytokine and chemokine profiles differ in accordance with various combinations of pattern recognition receptors in immune cells and MAMPs in Lactobacillus [37–39]. Previous in vivo studies have reported that Lactobacillus species elicit immune enhancement effects in various ways [40], with L. casei, L. rhamnosus, and L. plantarum in particular known to induce the release of inflammatory (TNF-α, IFN-γ, IL-12) and regulatory cytokines (IL-4, IL-10) through interactions with antigen-presenting cells in the intestine [13]. L. reuteri SKKU-OGDONS-01

treatment significantly increased the expression of antiviral cytokines IFN-β and IFN-γ (Fig. 7). However, we tested only representative cytokines in this study, so further study is needed to determine how this Lactobacillus strain elicits its immune enhancement effects.

Because we found that L. reuteri SKKU-OGDONS-01 increased the levels of antiviral cytokines, we expected it would have antiviral effects in the MNV-infection experiments. We administered the Lactobacillus for 2 weeks, so we selected MNV CR6, a viral strain that remains in vivo for a relatively long time [41]. The antiviral effect mediated by L. reuteri SKKU-OGDONS-01 was highest on the 7th day post-infection. The lowest level of viral protein was detected in mice treated with L. reuteri SKKU-OGDONS-01 compared with those who received L. paracasei ATCC 334 and L. reuteri KACC 11452 (Fig. 8).

To support the results from our in vivo experiments, we carried out data mining using transcriptome information and discovered probiotic markers. The stress-related genes that were highly expressed were mainly related to long-term acid stress resistance (Fig. 9). Furthermore, some virulence factors (dltD, sortase, dltA, and hemolysin III) involved in adherence in the gut were also discovered. These results support the findings from our in vivo system.

In conclusion, this study combined existing research methods such as in vivo mouse and in silico system to lay a foundation for more in-depth verification of the safety of new probiotics. Although probiotics have been proven safe many times, unconditional development is a dangerous approach because cases of infection have been reported. Safety should be verified using scientific approaches, and development of databases now permits cheap and efficient analysis. L. reuteri SKKU-OGDONS-01, which we verified for safety, could be used as a probiotic or as a tool for delivering specific antigens or proteins.

Conclusions

Although probiotics have been proven safe many times, unconditional development is a dangerous approach because cases of infection have been reported. Safety should be verified using scientific approaches, and development of genome and transcriptome databases now permits cheap and efficient analysis. L. reuteri SKKU-OGDONS-01, which we verified for safety, could be used as antiviral feed additives or as a tool for delivering specific antigens or proteins.

Methods

Sample collection and isolation of intestinal bacteria

The digestive tract of a 48-week-old female white leghorn chicken (provided by the Rural Development Administration, RDA, Republic of Korea) was harvested and divided into three parts (small intestine, cecum, and large intestine). Before isolating intestinal bacteria, the tissues were conserved in phosphate-buffered saline (PBS) supplemented with 80% glycerol. After homogenizing the intestinal tissues with 1.6 mm stainless steel beads (NEXT ADVANCE, USA), we diluted the PBS containing the extracted tissues to 1:100, spread them on MRS (de Man, Rogosa, and Sharpe) plates, and incubated them anaerobically at

37 °C for 2 days to isolate Lactobacillus strains. Colonies on the plates were collected randomly, using microscopy to choose only rod-shaped bacteria, and cultured in MRS medium at 37 °C. All isolated bacteria were maintained in MRS medium containing 80% glycerol. Control probiotic strains, Lactobacillus paracasei ATCC 334 (cheese-origin) and Lactobacillus reuteri KACC 11452 (human-origin), were kindly provided by Dr. Jos Seegers (Falcobio, Netherlands) and the RDA (Republic of Korea), respectively.

Primer design

All primers used are specified in Table 1. Universal bacterial primers were used to identify Lactobacillus species as previously reported [42]. The reference sequences used to detect various cytokines (IFN-β, IFN-γ, IL-6, and TNF-α) and the MNV capsid protein were obtained from GenBank (www.ncbi.nlm.nih.gov). The sets of cytokine primers and the murine norovirus detection primers were designed using the Primer 3 program.

Microbial identification using 16S rRNA sequences

Identification of bacterial isolates was performed using the 9F and 1542R primers, as previously reported by Yoon et al.[43]. The 16S ribosomal RNA (rRNA) sequences were amplified using a thermal cycler (Bio-Rad T100™ thermal cycler). PCR products were purified with a Zymoclean™ Gel DNA Recovery Kit (ZYMO RESEARCH, USA). The 16S rRNA sequences were obtained using the Sanger/capillary sequencing method (Macrogen, Republic of Korea). Taxonomic assignment for bacterial identification was conducted using the EzTaxon-e database and Basic Local Alignment Search Tool.

Construction of phylogenetic tree

The 16S rRNA gene sequences of an average length of 1500 bp were obtained from the NCBI database, and alignment was performed using the MEGA program (version 7). To confirm whether the isolates belong to same clade as similar Lactobacillus reuteri strains, various Lactobacillus reuteri strains (L. reuteri strain ZLR003, L. reuteri I5007, L. reuteri ATCC 53608, L. reuteri I49, L. reuteri JCM 1112, L. reuteri DSM 20016, L. reuteri IRT, and L. reuteri TD1) and L. fermentum IFO 3956, L. brevis ATCC 367, L. plantarum WCFS1, L. salivarius UCC118, L. rhamnosus GG, L. paracasei ATCC 334, L. iners DSM 13335, L. johnsonii NCC 533, L. jensenii JV-V16, L. delbrueckii subsp. bulgaricus ATCC 11842, L. crispatus ST1, L. helveticus CNRZ32, and L. acidophilus NCFM were compared. The phylogenetic relationships were determined based on the Neighbor-Joining method and bootstrap values (10000 replicates).

Preparation of genomic DNA and whole-genome sequencing

The genomic DNA of L. reuteri SKKU-OGDONS-01 was prepared, and whole-genome sequencing was conducted as previously reported by Kim et al.[44]. In brief, Lactobacillus was treated with a combination of lysozyme (20 mg/ml) and Mutanolysin (50 U/ml) in Tris-EDTA buffer at 37 °C for 1 hour for lysis of the bacterial cell wall. Purified 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. DNA

concentrations were determined by absorbance at 260 nm using a spectrophotometer. For whole-genome sequencing, only genomic DNA samples with an A260/A280 ratio of 1.8 or higher were collected because that was the cleanest band on the agarose gel. To construct a complete genome, both the PacBio RS II (Pacific Biosciences, USA) and Illumina Hiseq 2000 (Illumina, Inc., USA) platforms were used at Macrogen (Seoul, Republic of Korea). The genome assembly process was conducted in two stages: de novo assembly of long-reads generated from PacBio sequencing was carried out using SMRT Analysis software version 2.3.0 [45], and hybrid assembly using short-reads generated by Illumina sequencing was performed with Pilon (v1.21) to compensate for the high error rate of long-reads. Annotation of the genome was carried out using Prokaryote Genome Annotation Pipeline (PGAP) version 4.6 [46]. The complete genome of L. reuteri SKKU-OGDONS-01 was deposited at GenBank under accession number CP029615. The raw sequencing data can be accessed through BioProject number PRJNA473291 and BioSample number SAMN09270376.

Comparative analysis

Using the NCBI genome database (http://www.ncbi.nlm.nih.gov/genome), the genomes of 9 L. reuteri strains (L. reuteri DSM 20016, L. reuteri IRT, L. reuteri JCM 1112, L. reuteri I49, L. reuteri TD1, L. reuteri ATCC 53608, L. reuteri ZLR003, L. reuteri I5007, and L. reuteri SD2112) were retrieved and determined for comparative analysis. The similarity within the Lactobacillus genome was compared using the OrthoANI algorithm [47] and visualized using OrthoANI.

Mice, virus, and cells

6-week old BALB/c mice (DBL, Republic of Korea) were used for safety evaluation of Lactobacillus isolates, and 6-week old C57BL/6 mice (DBL, Republic of Korea) were used for the anti-MNV test.

All mice underwent adaptation for 1 week before the main experiment and were raised under standard laboratory conditions. All animal experiment procedures were performed following an approved animal-use document and according to the guidelines of the Institutional Animal Care and Use Committee of the National Institute of Animal Science (Approval No: 2018 – 276), Republic of Korea. RAW264.7 cells, which were used to proliferate the MNV, were cultivated in Dulbecco's modified Eagle's medium (Hyclone, USA) supplemented with 10% heat-inactivated fetal bovine serum (Alphabioregen, USA), 100 units/ml of penicillin, 100 μg/ml of streptomycin, 10 mM HEPES, and 2 mM glutamine at 37 °C in a 5% CO2 incubator. MNV GV/CR6/2005/USA was kindly provided by Herbert W. Virgin from Washington University [48].

Marker for measuring intestinal persistence of Lactobacillus

A modified vector system was used to measure the intestinal persistence of Lactobacillus by replacing the xylose-inducible promoter in the pSLP111.3 vector with the lactate dehydrogenase (LDH) constitutive promoter as previously reported [9]. Transformation of the vector system into the Lactobacillus was

carried out as described in a previous paper [9]. The original pSLP111.3 vector system was provided by Dr. Jos Seegers (Falcobio, Netherlands).

Measurement of persistence of selected Lactobacillus strains in the murine intestine

To determine the intestinal persistence and survivability of L. reuteri SKKU-OGDONS-01, a Lactobacillus isolated from chickens, we manipulated the following conditions: 1) whether antibiotics were given during the experiment and 2) the number of doses of Lactobacillus fed to the subjects. All mice in the antibiotic treatment group were given water containing 3 μg/ml chloramphenicol for 24 hours to remove some of the commensal bacteria from their intestines in the expectation that that would allow the transgenic Lactobacillus to be colonized in the intestine efficiently. Food and water were withdrawn 18 hours before Lactobacillus administration and returned after 10^8 colony forming units (CFUs) of Lactobacillus (L. paracasei ATCC 334 or L. reuteri SKKU-OGDONS-01) was administered using a feeding needle (20 gauge). One or three doses were given, and mice not receiving antibiotics were tested without any treatment before the experiment. In all mice, 1 g of feces was sampled daily, homogenized with stainless-steel beads in PBS, and serially diluted. The diluted fecal samples were spread on MRS plates containing 3 μg/ml chloramphenicol and incubated anaerobically at 37 °C for 2 days. In the negative control group, mice received only PBS instead of Lactobacillus under the same conditions. CFUs were calculated using the plate counting method.

Incidence of bacterial translocation

Bacterial translocation, in which administered bacteria move from the small intestine to blood and other organs, was evaluated as previously reported [49]. After the liver and kidneys were extracted in sterile conditions, each organ was homogenized with stainless steel beads in PBS. Blood was collected through the abdominal aorta, and the homogenized organ samples (100 μl and 20 μl blood) were plated on MRS agar or BHI (Brain Heart Infusion) agar (BD Biosciences, USA). After incubation at 37 °C for 48 hours, the plates were examined for viable colonies.

Immune-boosting effect of Lactobacillus in the gastrointestinal tract

After daily administration of Lactobacillus for 2 weeks, mice were sacrificed to harvest intestinal samples. The immune-boosting effect mediated by Lactobacillus was investigated using the mRNA expression levels of cytokines related to innate immunity. Total RNA was extracted with TRI reagent (Molecular Research Center, Inc., USA), and cDNA was produced for mRNA using the oligo dT primer and Moloney murine leukemia virus reverse transcriptase (Bioneer, Republic of Korea) according to the instructions. We produced cDNA using 5 μg of RNA and conducted real-time PCR using SYBR Premix Ex Taq (Takara, Japan) and a Rotor-Gene Q system (Qiagen, Australia) to quantitatively measure the levels of cytokine expression. Data analysis was conducted using Rotor-Gene Q series software version 2.3.1 (Qiagen, Australia).

Antiviral effect on MNV CR6 strain

Six-week-old MNV-seronegative male C57BL/6 mice were used to test the antiviral effect of L. reuteri SKKU-OGDONS-01 on MNV. To compare the efficacy of the chicken-originated Lactobacillus, cheese-originated L. paracasei ATCC 334 and human-originated L. reuteri KACC 11452 were used as control probiotics. Using the same scheme as in the immune-boosting experiments described above, 10^8 CFUs of Lactobacillus were administered daily for 2 weeks, and 10^5 plaque-forming units (PFUs) of MNV were given on the 15th day. After viral infection, Lactobacillus strains continued to be administered to the mice. Fecal sampling was conducted daily, but the samples used in the analysis were from days 5, 7, 9, and 14 after viral infection. To compare the antiviral effects of the Lactobacillus strains, we quantitatively compared the amounts of MNV capsid protein in the feces. Total RNA in the feces was isolated using a Quick-RNA™ Fecal / Soil Microbe Microprep Kit (ZYMORESEARCH, USA). cDNA was produced from 5 μg of total RNA with CellScript cDNA Synthesis Master Mix (CellSafe, Republic of Korea), and we compared the viral titer among experimental groups using quantitative real-time PCR as described above.

Transcriptome sequencing of L. reuteri SKKU-OGDONS-01

After incubating L. reuteri SKKU-OGDONS-01 in a pH7.0 environment to mimic its original niche, total RNA was harvested using a lysis mixture (20 mg/ml lysozyme (Sigma-Aldrich, USA) and 50 U/ml Mutanolysin from Streptomyces globisporus ATCC 21553 (Sigma-Aldrich, USA)) and a NucleoSpin® RNA Kit (MACHEREY-NAGEL, Germany) according to the manufacturer's instructions. Through RNA electrophoresis, two distinct bands representing 16S and 23S rRNA were identified, and only RNA samples with an A260/280 ratio greater than 1.8 were collected for transcriptome sequencing. To eliminate false RNA sequencing values caused by DNA, DNase was added. Then, the mRNA was purified using a Ribo-Zero rRNA Removal Kit (Bacteria) (Illumina, USA) to completely remove rRNA from the samples, followed by use of a TruSeq RNA Sample Prep Kit v2 for library preparation according to the manufacturer's instructions. Paired-end libraries (200bp–400 bp insert sizes) were sequenced using a HiSeq2000 (Macrogen, Republic of Korea).

Reads mapping and fragments per kilobase of transcript per million (FPKM) calculation

The RNA-seq raw data (4 fastq files) generated by Illumina sequencing (Illumina Hiseq2000 platform) were deposited in GenBank under accession number SRR9852143. Read trimming was performed using Trimmomatic 0.32 software in the default condition. Mapping of many sequencing reads to a reference genome (L. reuteri SKKU-OGDONS-01) was conducted using BBMap (short-read aligner) with the default parameters. After the mapping process, data mining for probiotic-specific markers was carried out based on the literature.

Statistical analysis

All experimental results were assessed using GraphPad Prism (GraphPad software version 5) as mean ± SEM. One-way ANOVA and t-test were applied for statistical analysis.

List Of Abbreviations

MNV Murine norovirus

IFN Interferon

TNF Tumor necrosis factor

MRS de Man, Rogosa, and Sharpe

scFv single chain variable fragment

LDH Lactose dehydrogenase

ALT Alanine aminotransferase

AST Aspartate aminotransferase

Declarations

Ethics approval and consent to participate

All applicable international and national guidelines for the care and use of chickens and mice were followed. All animal experiment procedures were performed following an approved animal-use document and according to the guidelines of the Institutional Animal Care and Use Committee of the National Institute of Animal Science (Approval No: 2018-276), Republic of Korea.

Consent for publication

Not applicable

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Availability of data and materials

The complete genome of *L. reuteri* SKKU-OGDONS-01 was deposited at GenBank under accession number CP029615. The raw sequencing data can be accessed through BioProject number PRJNA473291 and BioSample number SAMN09270376. The RNA-seq raw data (4 fastq files) generated by Illumina sequencing (Illumina Hiseq2000 platform) were deposited in GenBank under accession number SRR9852143.

Competing interests

The authors declare that they have no conflict of interest.

Authors' contribution

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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uncultured species. *International journal of systematic and evolutionary microbiology* 2012, **62**(3):716-721.

Figures

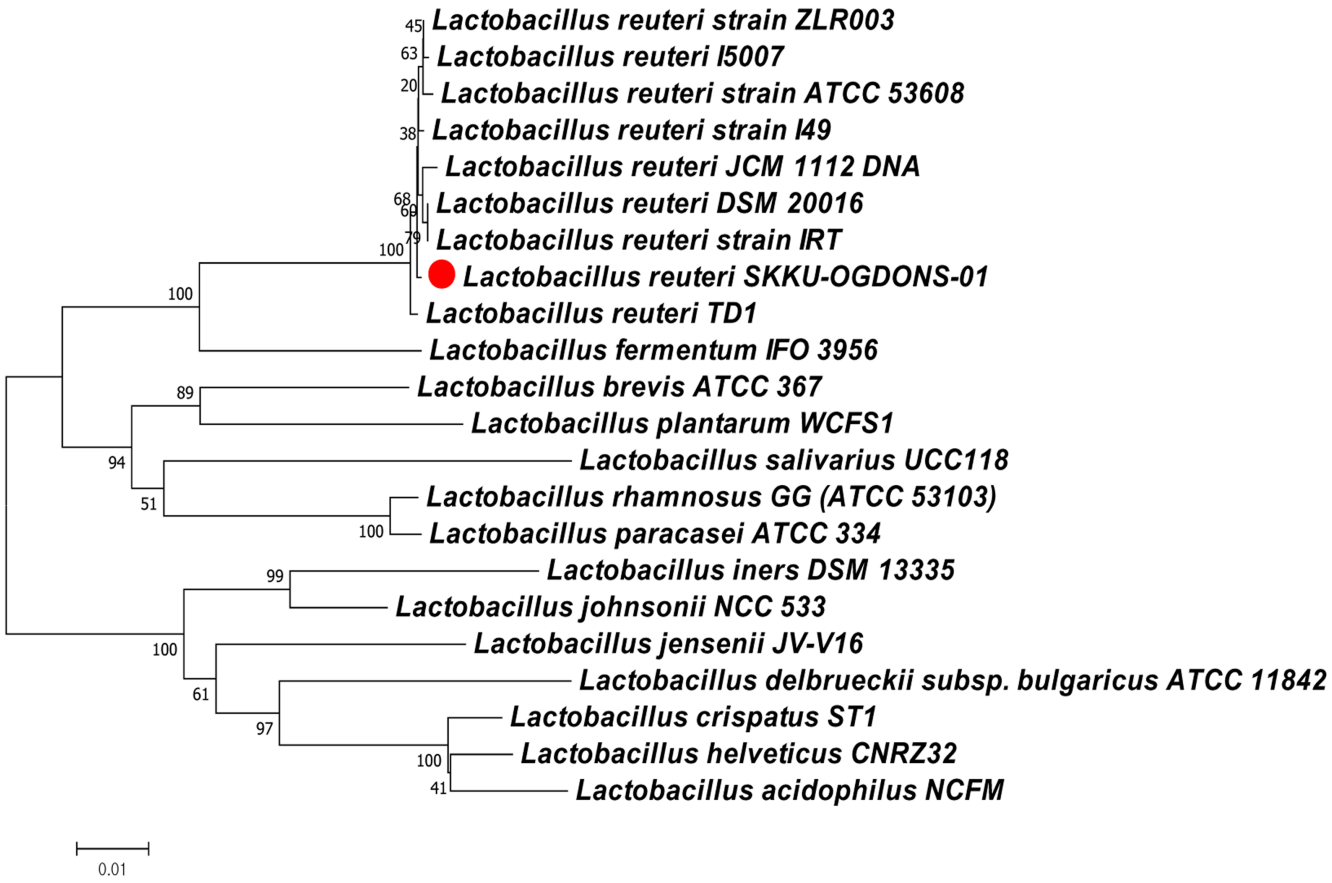

Figure 2

Construction of a phylogenetic tree based on the 16S rRNA sequences of Lactobacillus. A phylogenetic tree was constructed using the Neighbour-Joining method. The Lactobacillus species used were: various L. reuteri strains (including L. reuteri SKKU-OGDONS-01), 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 16S rRNA sequences of the reference strains were 1500 bp in length and were retrieved from NGBI GenBank. The tree was made using bootstrap values (10000 replicates). All values were greater than 40%. The scale represents 0.1 nucleotide substitution per position.

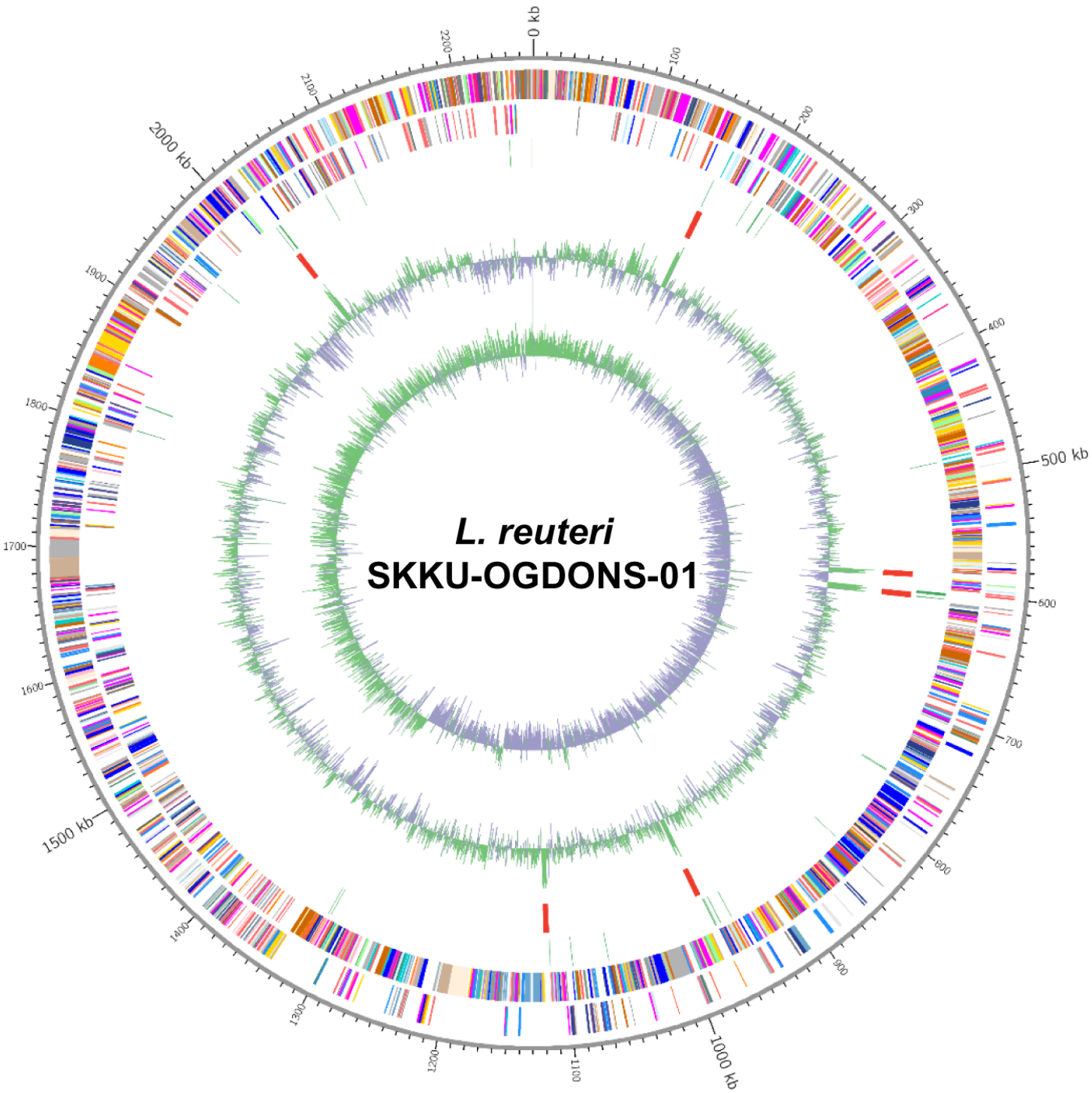

Figure 4

Chromosome map of the L. reuteri SKKU-OGDONS-01 genome. Representation of a circular chromosome map of Lactobacillus reuteri SKKU-OGDONS-01. Marked characteristics are displayed from the outside to the center: CDS on the forward strand, CDS on the reverse strand, tRNA (light green), rRNA (red), GC content, and GC skew. This map was visualized using circos.

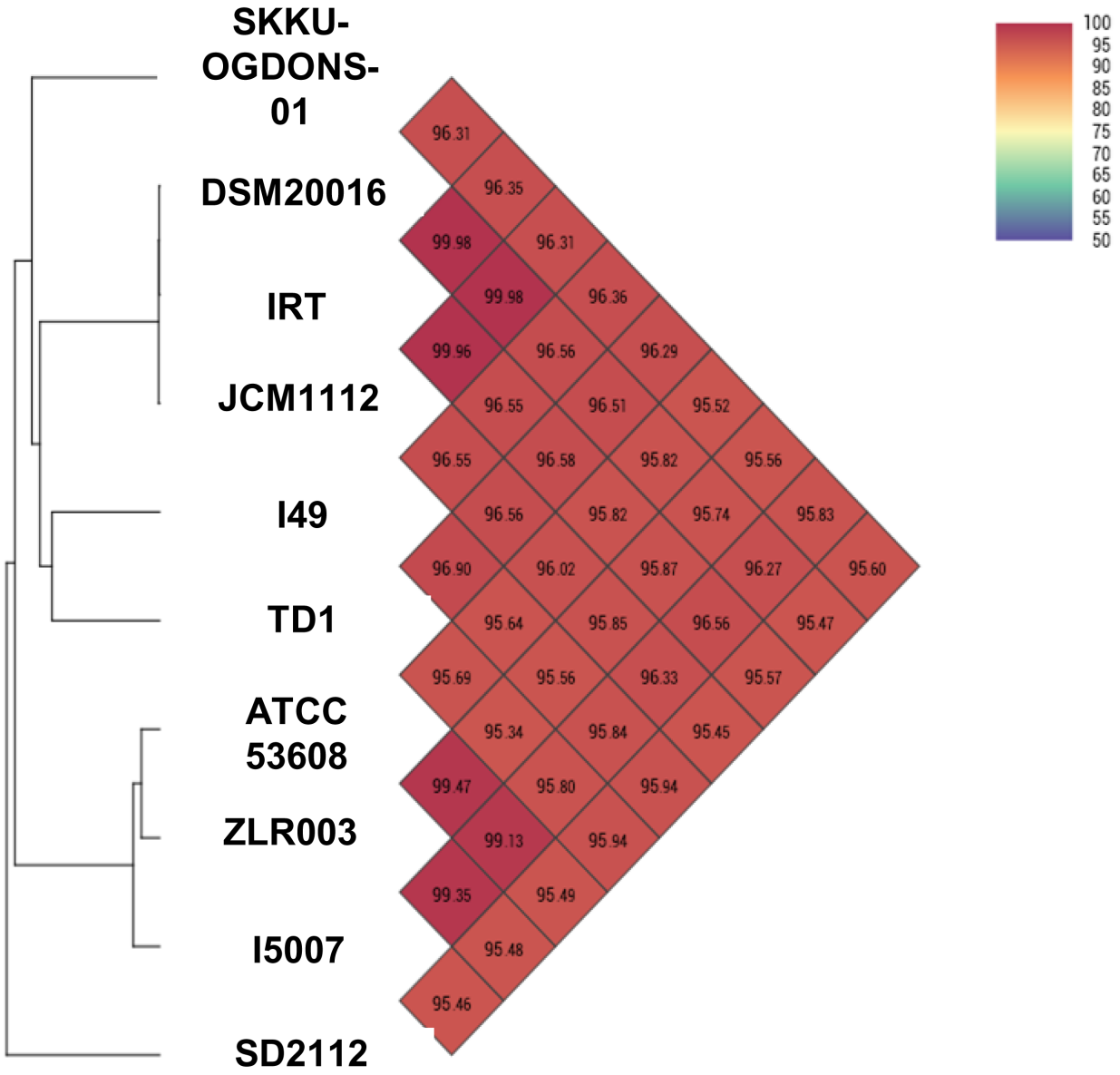

Figure 5

Comparative genome analysis using whole-genome sequences. This ANI phylogenetic tree was constructed based on OrthoANI values. Similarities between two genomes are marked next to the phylogenetic tree.

Figure 7

Schematic representation of the vector used to determine the retention time of L. reuteri SKKU-OGDONS-01 in the small intestine.

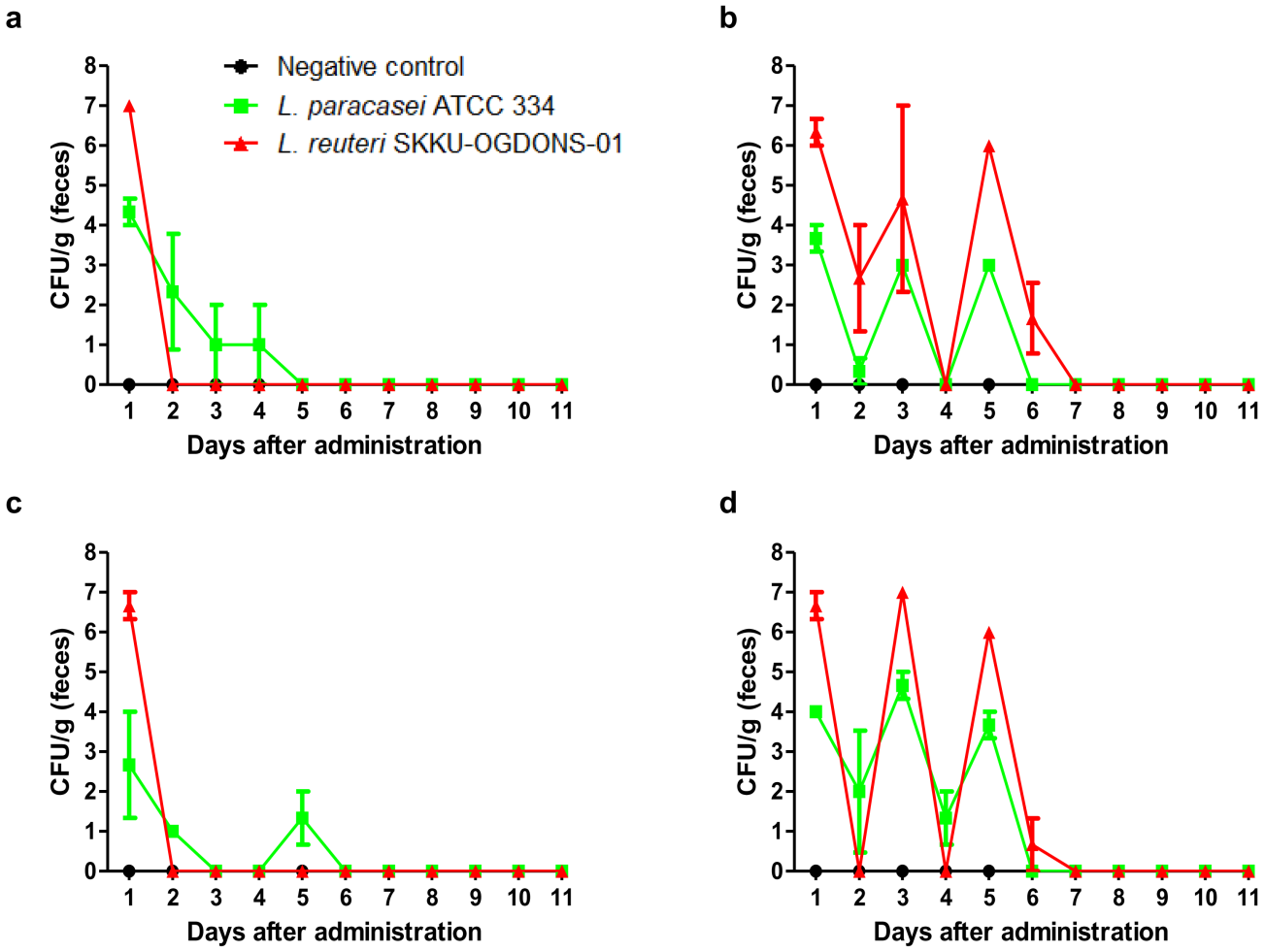

Figure 10

Survival and persistence of L. reuteri SKKU-OGDONS-01 in the gastrointestinal tracts of mice. Under the antibiotic treatment conditions, Lactobacillus strains (L. paracasei ATCC 334 and L. reuteri SKKU-OGDONS-01) were administered once (A) and three times every two days (B) at a dose of 108 CFUs. In the absence of antibiotics, Lactobacillus strains (L. paracasei ATCC 334 and L. reuteri SKKU-OGDONS-01) were administered once (C) or three times every two days (D). Data are presented as the mean ± SEM. (●: Negative control, ■: L. paracasei ATCC 334, ▲: L. reuteri SKKU-OGDONS-01)

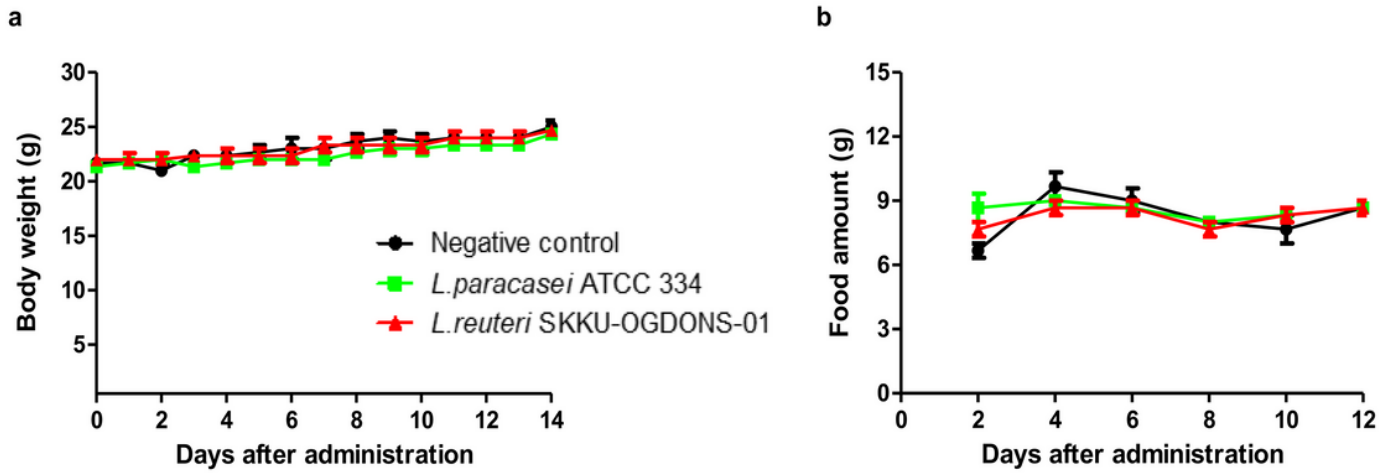

Figure 12

Monitoring weight changes and food intake during Lactobacillus administration. L. paracasei ATCC 334 and L. reuteri SKKU-OGDONS-01 were administered to mice daily for 2 weeks. During the administration period, the body weights of the mice were monitored daily (A). Total food intake (B) during administration is indicated on the graph. Data are presented as the mean ± SEM for n=3.

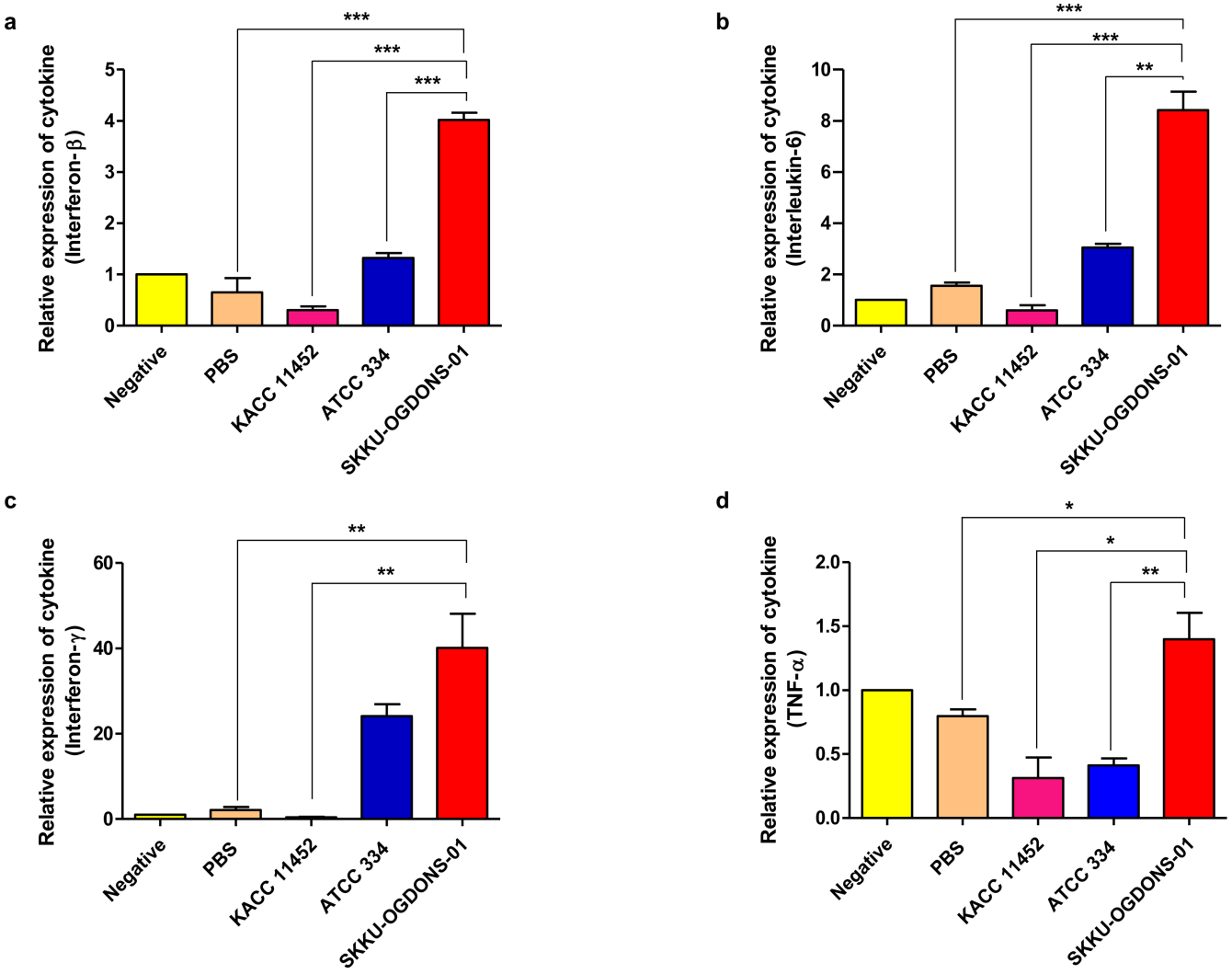

Figure 14

Immune-boosting effects of L. reuteri SKKU-OGDONS-01. The expression of cytokines induced by Lactobacillus strains was measured using qRT-PCR analysis. The immune-boosting activity of L. reuteri KACC 11452 (originating from humans) was compared with that of L. reuteri SKKU-OGDONS-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 cytokine expression induced by the Lactobacillus strains was calculated using the delta delta CT method. Data are presented as the mean ± SEM for n=3. *p<0.05, **p<0.01.

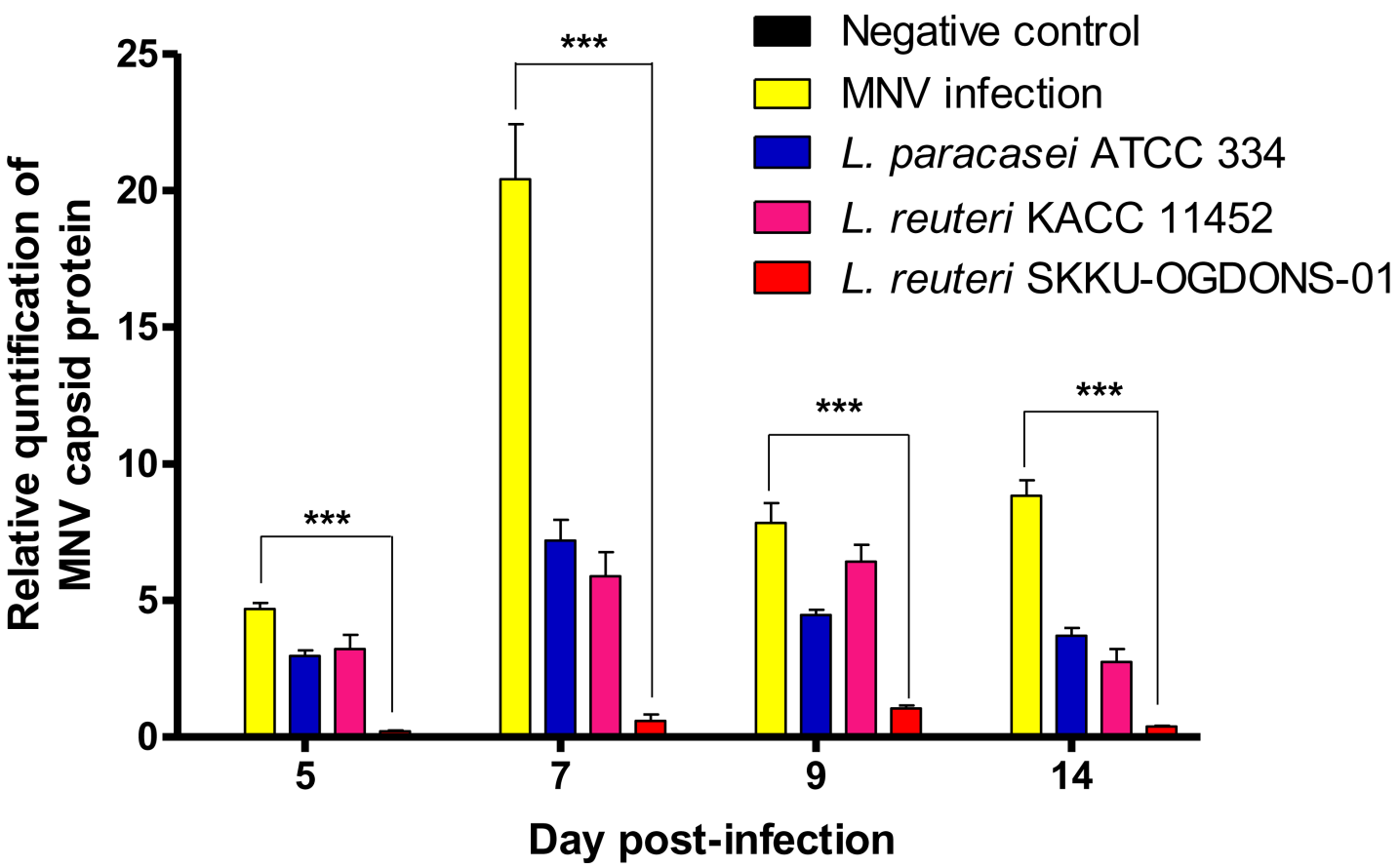

Figure 16

Antiviral efficacy of L. reuteri SKKU-OGDONS-01 against murine norovirus CR6. Before viral infection, Lactobacillus strains (L. paracasei ATCC 334, L. reuteri KACC 11452, and L. reuteri SKKU-OGDONS-01) were administered daily for 2 weeks, and then the antiviral effect of the Lactobacillus strains was observed in MNV-infected mice. Fecal samples from days 5, 7, 9, and 14 after infection were used to compare the amounts of viral protein among conditions. The relative expression of the MNV capsid protein in the 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 ± SEM for n=3. **p<0.01, ***p<0.001

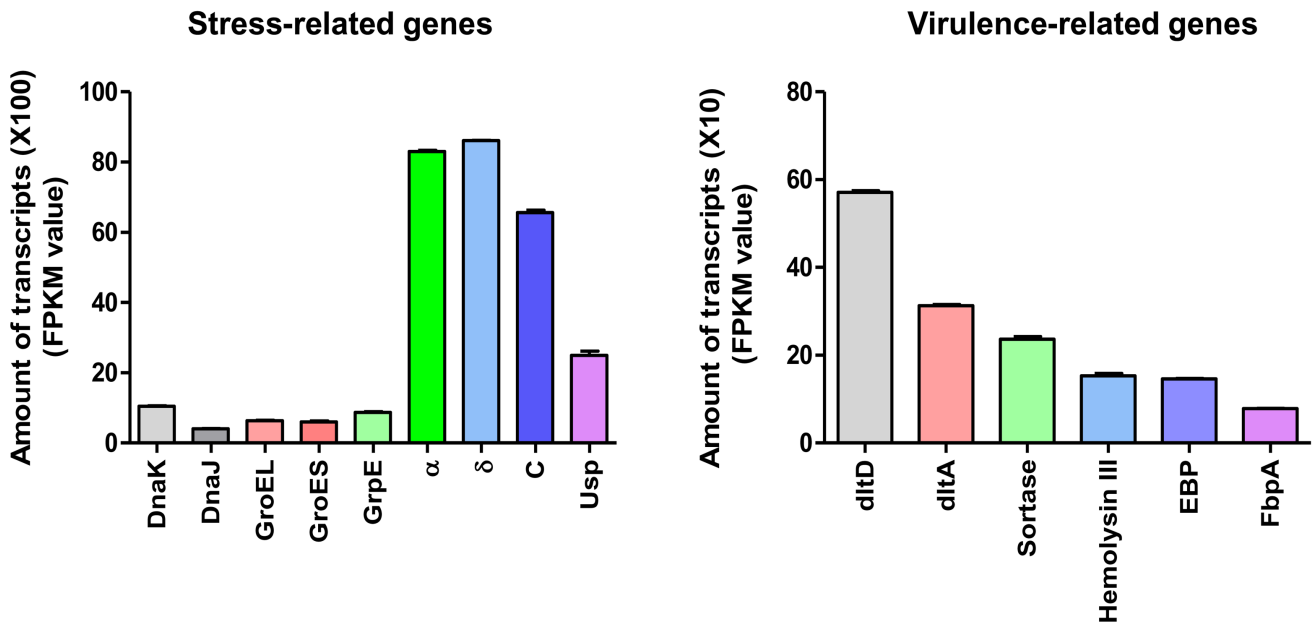

Figure 18

Expression levels of probiotic markers in L. reuteri SKKU-OGDONS-01. Through data mining, probiotic markers were discovered in the transcriptome of L. reuteri SKKU-OGDONS-01. The expression level for each gene is indicated in terms of FPKM (Fragment Per Kilobase of transcript per Million mapped reads). Data are presented as the mean ± SEM for n=2.