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Safety assessment of *Lactobacillus reuteri* IDCC 3701 based on phenotypic and genomic analysis

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Abstract

Purpose: *Lactobacillus reuteri* is one of the most largely studied human-commensal bacteria and widely used as a form of probiotics. Safety of probiotics has become increasingly important for human consumption due to increasing health-concerns in food industry. In this study, the safety of *L. reuteri* IDCC 3701 isolated from human breast milk was thoroughly investigated.

Methods: Whole-genome sequence analysis was performed to identify antibiotic resistance and toxigenicity of *L. reuteri* IDCC 3701. Phenotypic analysis such as minimal inhibitory concentration, β-hemolysis, extracellular enzyme activity, and the production of biological amines and L/D-lactate, was investigated. Finally, acute oral toxicity test was performed to access *L. reuteri* IDCC 3701 safety.

Results: Although multiple resistances to gentamicin and kanamycin were observed in *L. reuteri* IDCC 3701, it was revealed that these resistances are intrinsic and not transferable through whole-genome analysis. In addition, various phenotypic analysis concerning hemolysis, enzyme activity, and D-lactate production did not show any negative results. Although *L. reuteri* IDCC 3701 harbors a histidine decarboxylase gene, no biogenic amines were detected. Finally, *L. reuteri* IDCC 3701 exhibited no evidence of acute toxicity according to an in vivo study.

Conclusion: Our findings demonstrate that *L. reuteri* IDCC 3701 is considered to be safe for human consumption as probiotics based on the in silico, in vitro and in vivo studies.

Keywords: Probiotics, Lactic acid bacteria, *Lactobacillus reuteri*, Safety evaluation

Introduction

Probiotics have recently begun to be considered as one of the most effective sources of functional foods (Byakika et al. 2019; Saarela 2019). Typically, *Lactobacillus*, *Bifidobacterium*, and *Saccharomyces* strains are representatives found in probiotic formulations for commercial markets (Ricci et al. 2017). Although these probiotics are prevalent in the intestine as beneficial microorganisms, they can cause adverse events such as inflammatory bowel disease in rare case (Doron and Snydman 2015). Thus, the safety evaluation of probiotic strains has become the most fundamental criterion for human consumption in the food industry (Salvetti et al. 2016). Especially, the safety of new isolated species cannot be equal to that of pre-existing species, because each of probiotic strain has strain-specific characteristics (Alayande et al. 2020; Salvetti et al. 2016). Thus, individual strain should be evaluated for safety as probiotics independently.

Lactobacillus reuteri is an obligate heterofermentative species and has been widely reported to confer multiple beneficial effects by improving gut mucosal integrity and nutrient absorption (Hou et al. 2015; Mu et al. 2018;

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Zheng et al. 2020). Additionally, *L. reuteri* has been found in a variety of hosts, including human and animals (Duar et al. 2017), and also in various body parts, including the gastrointestinal tract and breast (Böttcher et al. 2008; Valeur et al. 2004), implying that *L. reuteri* could be one of the most prevalent and fundamental probiotic strains.

In this study, potential risks of *L. reuteri* IDCC 3701, isolated from breast milk, were assessed by genomic and phenotypic analysis as well as oral acute toxicity study. As genomic analysis, antibiotic resistance genes, virulence genes and mobile elements were identified. As phenotypic analysis, hemolytic activity, enzymatic activity, biogenic amines and L-/D-lactate production, and minimum inhibitory concentrations (MICs) were investigated. Thus, this study provides a comprehensive outline for safety assessment of *L. reuteri* strain.

Materials and methods

L. reuteri IDCC 3701

To isolate bacteria that producing lactate, diluted breast milk samples (5 mL × 3) were spread on MRS medium (BD Difco, Frankilin Lakes, NJ, USA) containing 0.5% (w/v) $CaCO_3$ in a static incubator (IST-4075R, SANYO, Osaka, Japan) with 0.5% CO_2 at 37 °C. Then, we identified *L. reuteri* IDCC 3701 (ATCC BAA-2837) by 16s rRNA sequencing (GenBank no. KM453732) of a colony forming hollow. The strain was maintained MRS medium and stored in 20% (w/w) glycerol at − 80 °C for further usage.

Identification of antibiotic resistance, virulence genes, mobile elements, and biogenic amines relate genes

The whole-genome sequencing of *L. reuteri* IDCC 3701 was performed using a PacBio RSII instrument with an Illumina platform (Macrogen, Seoul, Korea). A nucleotides-sequence was generated by single molecule real-time (SMRT) sequencing system. Contigs were constructed by pre-assembling seed reads, by generating a consensus sequence of the mapped reads, and by correcting and filtering the reads. A consensus sequence with higher quality was obtained after error-correction of the constructed contigs by Pilon (version 1.21).

Putative virulence factors were identified with the BLASTn algorithm using the virulence factor database (VFDB; version 2020.02.13; http://www.mgc.ac.cn/VFs/) with identity > 70%, coverage > 70%, and *E* value < 1E−5 (Chen et al. 2005). For putative antibiotic resistance genes, the assembled sequences were compared to the reference antibiotic resistance gene sequences in the ResFinder database (https://cge.cbs.dtu.dk/services/ResFinder/) using ResFinder 3.2 software. The search parameters for the analysis were sequence identity > 80% and coverage > 60% (Zankari et al. 2012). Prophage regions were identified using PHASTER web-based program (Arndt et al. 2016). Transposases and conjugal transfer proteins were annotated using the BLASTP against on transposases and conjugal transfer proteins retrieved from GenBank. The genomic islands were predicted using the GIPSy program (Soares et al. 2016).

It has been reported that histidine decarboxylase, tyrosine decarboxylase, lysine decarboxylase, ornithine decarboxylase, phenylalanine decarboxylase, and the enzymes involved in agmatine deiminase pathway such as N-carbamoylputrescine amidase and agmatinase were the key enzymes for biosynthesis of biogenic amines (Gardini et al. 2016). After building profile hidden Markov models (HMMs) representing the conserved amino acid sequence patterns in these enzymes, the candidate biogenic amine biosynthesis genes on the IDCC 3701 genome were searched using the hmmsearch tool in HMMER package (Eddy 2011) with the constructed profile HMMs. The candidate genes were confirmed using BLASTP tool in NCBI BLAST+ and hmmscan tool in HMMER package against SWISS-PROT database and PFAM database, respectively.

Determination of minimal inhibitory concentrations (MICs)

L. reuteri IDCC 3701 was assessed for susceptibility to a variety of antibiotics including ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline, and chloramphenicol (Sigma-Aldrich, St. Louis, MO, USA), which are typically used to treat enterococcal infections (Wiegand et al. 2008). The plate was then incubated at 37 °C anaerobically for 18 h and the optical density was observed using a microplate reader (BioTek, Winooski, VT, USA).

β-Hemolytic and enzymatic activities

Overnight culture of *L. reuteri* IDCC 3701 was streaked on Sheep blood agar plates (BD Difco) and incubated at 37°C overnight. Then, the hemolytic potential of *L. reuteri* IDCC 3701 was evaluated by observing the cleared zones around the colonies. *Staphylococcus aureus* ATCC 25923 which was incubated in blood-based brain heart infusion (BHI) medium at 37 °C was used as a positive control for β-hemolysis by culturing on sheep blood agar plates.

Enzymatic activities were investigated using the API ZYM Kit (BioMérieux, Marcy-l'Etoile, France). In brief, 1.8×10^9 CFU/mL of *L. reuteri* IDCC 3701 was incubated in MRS medium at 37 °C for 4 h prior to loading into the API ZYM strips. Then, one drop of each of ZYM-A and ZYM-B reagents were added to each well. After 5 min, color changes were observed and compared to the color reaction chart.

Biogenic amines and D-/L-lactate production

Biogenic amines were measured according to Priyadarshani and Rakshit 2011. Briefly, the supernatant from overnight cultured *L. reuteri* IDCC 3701 was collected after centrifugation at 2300×*g* and 4 °C for 30 min and filtered with a 0.22-μm-pore size membrane. Prior to the quantification of the biogenic amines (BAs), 0.75 mL of supernatant was mixed with the equivalent of 0.1 M HCl and filtered with a 0.45-μm membrane to extract the BAs. Then, 1 mL of the extracted BAs was incubated in a water bath at 70 °C for 10 min, followed by the addition of 200 μL of saturated $NaHCO_3$, 20 μL of 2 M NaOH, and 0.5 mL of dansyl chloride (10 mg/mL acetone). The derivatized BAs were mixed with 200 μL of proline (100 mg/mL H2O) and incubated in the dark at room temperature for 15 min. Acetonitrile (HPLC grade; Sigma-Aldrich) was added to the mixture to a total volume of 5 mL. The derivatized BAs were separated and quantified by HPLC (LC-NETII/ADC, Jasco, Macclesfield, UK) using an Athena C18 column (4.6 mm × 250 mm, ANPEL Laboratory Technologies, Shanghai, China) and a UV detector (UV-2075 plus, Jasco, Macclesfield, UK) at 254 nm. Aqueous acetonitrile solution (67:33 of H_2O, v/v) was used as a mobile phase at a constant flow rate of 0.8 mL/min. Tyramine, histamine, putrescine, 2-phenethylamine, and cadaverine were used as standards.

For the analysis of lactate, the supernatant from overnight cultured *L. reuteri* IDCC 3701 was collected by centrifugation at 2300×*g* and 4 °C for 30 min and then filtered with a 0.22-μm-pore size membrane. The quantities of L- and D-lactate in the supernatants were measured using an assay kit (Megazyme, Bray, Ireland) according to the manufacturer's protocol.

Acute oral toxicity in rats

All the rats were bred in Korea Testing and Research Institute (KTR; Hwasun-gun, Jeollanam-do, Korea) facilities which was maintained in an ambient temperature of 20.6–23.4 °C with a relative humidity of 47.6–59.7% on an automatically controlled 12/12 light cycle (lights off at 20:00 h) using 150–300 Lux. Less than 3 rats were in (310 W × 50 D × 20 H) mm cage and rats had free access to food (Rodent Diet 20 5053; Labdiet, St. Louis, MO, USA) and sufficient water. All efforts were made to minimize animal suffering and distress and the number of animals required for the production of reliable scientific data. The animal experiments (acute oral toxicity (AOT) test) in this study were conducted by KTR (TGK-2020-000008) under Animal protection act (no. 14651) and Laboratory animal act (no. 15278) by Korea government. Among four groups of three female rats (two groups aged nine weeks and two groups aged 10 weeks), each group was orally treated with 10 mL of *L. reuteri* IDCC 3701 (13.8×10^{10} CFU/g) per kg body weight (BW) (i.e., 300 mg/kg BW or 2000 mg/kg BW). After observation of the clinical signs of morbidity, mortality, and BW during the 14 days (i.e., at the end of experimentation), animals were euthanized via isoflurane anesthesia.

Results and discussion

Whole genome analysis and determination of MICs

The whole-genome sequence of *L. reuteri* IDCC 3701 indicated that this strain harbors only chromosomal DNA without plasmid and the total length of the genome was about 2.09 million base pairs with a GC content of 38.87% and 2,087 functional genes (CDS) (Supplementary Fig. 1, Table 1).

For in silico safety analysis, we analyzed potential antibiotic resistance genes virulence factors, and mobile elements of *L. reuteri* IDCC 3701. On the basis of the BLASTn algorithm and the VFDB, no putative virulence gene was identified in *L. reuteri* IDCC 3701. In addition, no genes related to antibiotic resistance associated with aminoglycosides, beta-lactams, colistin, fosfomycin, fusidic acid, macrolides, nitroimidazole, oxazolidinone, phenicols, quinolones, rifampicin, sulphonamides, tetracyclines, trimethoprim, or glycopeptides were identified in the genome of *L. reuteri* IDCC 3701 (Table 2). Furthermore, mobile elements, such as transposes (64 genes), genomic island (15 genes), and prophage (0 gene) in the genome were analyzed in order to predict the possibility of antibiotic resistance gene transfer. However, these genes were meaningless due to absence of potential antibiotic resistance gene. Next, MICs of *L. reuteri* IDCC 3701 against nine antibiotics were analyzed to verify the safety of this strain. In results, *L. reuteri* IDCC 3701 was susceptible to all of the antibiotics, except for gentamicin and kanamycin (Table 2). The observed MICs for gentamicin and kanamycin were 2-fold and 4-fold dilution above the EFSA cut-off value, respectively.

In this study, *L. reuteri* IDCC 3701 exhibited to be susceptible to all of the antibiotics, except for gentamicin and kanamycin (Table 2). Thus, the resistance against gentamicin and kanamycin seems to be intrinsic traits of this strain. Indeed, many *Lactobacillus* species have been found to be relatively tolerant to aminoglycoside antibiotics, such as gentamicin, kanamycin, and streptomycin

Table 1 Genome information of *L. reuteri* IDCC 3701

Items	Results
Identification	*Lactobacillus reuteri*
ANI value (%)	99.997
No. of 16s rRNA	18
Genome size (bp)	2,085,171
GC contents (%)	38.87
CDS	2,087

Table 2 Minimal inhibitory concentrations and antibiotic resistance gene of *L. reuteri* IDCC 3701

Antibiotic[a]	AMP	VAN	GEN	KAN	STR	ERY	CLI	TET	CHL
Cut-off value[b] (μg/mL)	2	n.r[c]	8	64	64	1	4	32	4
Observed MIC	0.5	512	16	256	32-64	0.125-0.5	0.125-0.5	4	4
Assessment	S[d]	n.r	R[e]	R	S	S	S	S	S
Antibiotic resistance gene	n.d[f]	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d

[a]*AMP* ampicillin, *VAN* vancomycin, *GEN* gentamicin, *KAN* kanamycin, *STR* streptomycin, *ERY* erythromycin, *CLI* clindamycin, *TET* tetracycline, *CHL* chloramphenicol
[b] EFSA (European Food Safety Authority) Guidelines, 2018. EFSA Journal, 16(3), 5206
[c]*n.r* not required
[d]*S* susceptible
[e]*R* resistant
[f]*n.d* not detected

(Danielsen and Wind 2003). This intrinsic resistance mechanism species might be due to membrane properties of these bacteria (Campedelli et al. 2018). In conclusion, *L. reuteri* IDCC 3701 has demonstrated to be safety with regard to antibiotic resistance.

β-Hemolytic and enzymatic activities

β-Hemolytic activity, which is a representative virulence factor enrolling in pathogenic invasion process to a host, should be absent in a probiotic strain to ensure safety. In this study, *L. reuteri* IDCC 3701 showed no hemolytic activity (γ-hemolytic, no clear zone), while *S. aureus* ATCC 25923, a positive control, clearly showed β-hemolytic activity (a clear zone) on blood-based BHI agar (Supplementary Fig. 2). Enzyme activities involved in carbohydrate metabolism (α-galactosidase, β-galactosidase, and α-galactosidase), lipid metabolism (esterase), and vitamin metabolism (acid phosphatase) were detected in *L. reuteri* IDCC 3701 (Table 3). Although β-glucosidase hydrolyzes glucose conjugates to generate beneficial secondary metabolites in the colon, it was also reported to produce potential carcinogenic compounds in rare cases (Cole and Fuller 1987). Meanwhile, β-glucuronidase which might directly link to carcinogenic compounds and thereby increase risk for colorectal cancer (Kim and Jin 2001), were not detected in *L. reuteri* IDCC 3701.

Biogenic amines and lactate production

Biogenic amines (BAs) production has been reported in some lactic acid bacteria (Beneduce et al. 2010; Özogul and Özogul, 2019; Spano et al. 2010). Large quantity of BAs is regarded as a potential health risk factor due to their toxicological effects (Spano et al. 2010). Histamine and tyramine are responsible for food-induced migraine, hypertensive crisis, and scombroid food poisoning (histamine intoxication) (Özogul and Özogul, 2019). Thus, profile search and homology search were performed using HMMER and BLAST algorithms. The genome harbors one gene (IDCC3701_1_01385) encoding a histidine decarboxylase. The gene has the high similarity with the reference sequence (86.8% of P00862 from *Lactobacillus* sp. 30A (GenBank Acc. No. AAB59151)) that was experimentally confirmed (Vanderslice et al. 1986). However, it was confirmed that other biosynthesis enzymes containing the tyrosine decarboxylase were not presented in this genome. Furthermore, in the supernatant of *L. reuteri* IDCC 3701, no biogenic amines such as tyramine, histamine putrescine, cadaverine, and 2-phenethylamine were detected in this strain (data not shown).

Some lactic acid bacteria can produce a mixture of L- and D-lactate (Stiles and Holzapfel 1997). Although D-lactate is not a highly toxic compound, D-lactate accumulation in blood (i.e., 3 > mmol/L, Petersen, 2005) might cause health problems due to

Table 3 Enzymatic activities of *L. reuteri* IDCC 3701

Enzyme	*L. reuteri*
Alkaline phosphate	–[a]
Esterase	+[b]
Esterase lipase	–
Lipase	–
Leucine arylamidase	++[c]
Valine arylamidase	–
Cystine arylamidase	–
Trypsin	–
α-chymotrypsin	–
Acid phosphatase	++
Naphthol-AS-BI-phosphohydrolase	++
α-galactosidase	+++[d]
β-galactosidase	+++
β-glucuronidase	–
α-glucosidase	+
β-glucosidase	–
N-acetyl-β-glucosaminidase	–
α-mannosidase	–
α-fucosidase	–

[a] – no enzymatic activity
[b]+ poor presence of enzymatic activity
[c]++ moderate presence of enzymatic activity
[d]+++ high presence of enzymatic activity

Table 4 Body-weight changes of the rats administered *L. reuteri* IDCC 3701

Group	Dose (g/kg BW[a])	Day after administration				
		0	1	3	7	14
9-week-aged	300	193.0 ± 2.2	220.1 ± 6.0	225.0 ± 6.4	239.8 ± 13.0	254.9 ± 15.0
	2000	212.5 ± 10.6	231.5 ± 14.8	239.5 ± 16.5	250.1 ± 14.3	255.7 ± 18.6
10-week-aged	300	220.1 ± 7.9	220.9 ± 6.4	237.6 ± 9.9	257.5 ± 4.0	273.5 ± 3.3
	2000	217.3 ± 12.8	242.7 ± 15.9	249.5 ± 12.0	245.4 ± 17.5	251.5 ± 21.6

[a]*BW* body weight

a poor capacity of metabolizing D-lactate in human (Pohanka 2020; Puwanant et al. 2005). However, more recent studies have shown that D-lactate accumulation may only occur in cases of impaired D-lactate metabolism or a disturbed gastrointestinal function following any clinical symptoms, including short bowel syndrome (Connolly et al. 2005; Ku et al. 2006). In this study, *L. reuteri* IDCC 3701 was found to predominantly produces L-lactate (20.5 mg/mL, which is equivalent to 78.8%) rather than D-lactate (5.5 mg/mL, 21.2%), respectively.

Single-dose acute oral toxicity study

The in vivo toxicity of *L. reuteri* IDCC 3701 was investigated by a single-dose acute oral toxicity test. The results of the 14-day feeding to rats revealed that an oral administration of *L. reuteri* IDCC 3701 using 1.4×10^{10} CFU/g caused no abnormal findings in rats. In addition, there was no difference in gain-weight or loss of feed intake (Table 4), and also there was no change in appearance, behavior, or survival rate during the 14-day observation. At necropsy, no lesions due to the feeding of *L. reuteri* IDCC 3701 were detected in any organs. In sum, consumption of 0.3 g and 2 g of *L. reuteri* IDCC 3701 per kg BW of rat was not nontoxic to the tested rats.

Conclusion

The safety of *L. reuteri* IDCC 3701 was demonstrated by genomic and phenotypic analyses and acute toxicity studies. The strain was found to be negative for antibiotic resistance and toxigenicity genes in the genome analysis. The strain did not produce any toxic compounds according to the phenotypic analysis. Furthermore, oral administration of this probiotic strain to rats showed no hazardous phenomena. Therefore, *L. reuteri* IDCC 3701 isolated from breast milk can be considered safe for human consumption as a probiotic.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13213-021-01622-y.

Additional file 1: Figure S1. Circular genome map and function genes of *L. reuteri* IDCC 3701. **Figure S2.** No β-hemolytic activity of *L. reuteri* IDCC 3701.

Abbreviations

AMP: Ampicillin; BA: Biogenic amine; BHI: Brain heart infusion; CHL: Chloramphenicol; CLI: Clindamycin; ERY: Erythromycin; GEN: Gentamicin; HMM: Hidden Markov model; KAN: Kanamycin; MIC: Minimum inhibitory concentration; STR: Streptomycin; TET: Tetracycline; VAN: Vancomycin; VFDB: Virulence factor data base

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Authors' contributions

Bo Som Lee and O-Hyun Ban performed experiments and analyzed the data. Won Yeong Bang, Seung A Choi, and Sangki Oh helped to perform experiments and analyze the data. Minjee Lee and Chanmi Park helped to finalize the manuscript. Jungwoo Yang and Young Hoon Jung designed and wrote the manuscript. Young Hoon Jung supervised the project. Soo Jung Kim aided in interpreting the results and provided critical revision. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article, while 16S rDNA sequences of *L. reuteri* IDCC 3701 are deposited to NCBI Genome Information and available under the accession number, KM453732 (https://www.ncbi.nlm.nih.gov/nuccore/719239383/).

Ethics approval and consent to participate

Not applicable

Consent for publication

All authors read and approved the final manuscript for publication.

Competing interests

The authors declare that they have no competing interests.

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