INTRODUCTION

Probiotics can be defined as “live microorganisms, which when administered in adequate amounts confer a health benefit to the host” (FAO/WHO, 2002). To date, probiotics have become commercially available as nutritional supplements in the form of various food products (Plessas et al., 2012). Because microorganisms derived from food fermentation have been considered safe and are generally referred to as Generally Recognized As Safe (GRAS) microorganisms, the majority of lactic acid bacteria (LAB) and bifidobacteria can be generally considered as safe for use (Holzapfel et al., 1998; Leroy and Vuyst, 2004; Wessels et al., 2004). Nevertheless, probiotics, including newly isolated strains, need to be characterized in terms of more safety aspects to ensure they are safe for human consumption (Huys et al., 2013). An FAO/WHO working team suggested that consequence tests, including metabolic activities, antibiotic resistance, hemolytic activity, toxin production, infectivity, and metabolic activity in immune-compromised animal species. The results demonstrated that the strain was susceptible to nine antibiotics suggested by the European Food Safety Authority (EFSA). Whole-genome analysis revealed that L. lactis IDCC 2301 neither has toxigenic genes nor harbors antibiotic resistance. Moreover, L. lactis IDCC 2301 showed neither hemolytic nor β-glucuronidase activity. Furthermore, none of the D-lactate and biogenic amines were produced by L. lactis IDCC 2301. Finally, it was demonstrated that there was no toxicity and mortality using single-dose oral toxicity tests in rats. These results indicate that L. lactis IDCC 2301 can be safely used as probiotics for human consumption.

KEYWORDS

homemade cheese, Lactococcus lactis IDCC2301, probiotics, safety evaluation

1 INTRODUCTION

Probiotics can be defined as “live microorganisms, which when administered in adequate amounts confer a health benefit to the host” (FAO/WHO, 2002). To date, probiotics have become commercially available as nutritional supplements in the form of various food products (Plessas et al., 2012). Because microorganisms derived from food fermentation have been considered safe and are generally referred to as Generally Recognized As Safe (GRAS) microorganisms, the majority of lactic acid bacteria (LAB) and bifidobacteria can be generally considered as safe for use (Holzapfel et al., 1998; Leroy and Vuyst, 2004; Wessels et al., 2004). Nevertheless, probiotics, including newly isolated strains, need to be characterized in terms of more safety aspects to ensure they are safe for human consumption (Huys et al., 2013). An FAO/WHO working team suggested that consequence tests, including metabolic activities, antibiotic resistance,
2.3 | Examination of antibiotic susceptibility

The susceptibility of L. lactis IDCC 2301 to various antibiotics, which are commonly used for the treatment of enterococcal infections, was evaluated. In this test, nine antibiotics were used, namely ampicillin, clindamycin, chloramphenicol, erythromycin, gentamicin, kanamycin, streptomycin, tetracycline, and vancomycin. In brief, the cells were grown overnight, and then, 1% (v/v) of cells was transferred to fresh MRS broth. When the cell density reached approximately $10^8$ CFU/mL, 50 μL of culture broth and 50 μL of each antibiotic solution at various concentrations were transferred to a 96-well plate to obtain $3.3–6.6 \times 10^5$ CFU/mL and 0.125–1024 μg/mL, respectively. Then, the plates were incubated at 37°C for 20 h, and the absorbance at 600 nm was measured using a microplate spectrophotometer (BioTek). Finally, MIC was determined as the lowest concentration in which cell growth was completely inhibited.

2.4 | Genomic analysis of virulence factors and antibiotic genes in L. lactis

A PacBio RSII instrument with an Illumina platform (Macrogen) was used for whole-genome sequencing of L. lactis IDCC 2301. Putative virulence factors were identified using the BLASTn algorithm based on the virulence factor database (http://www.mgc.ac.cn/VFs/) setting thresholds of identity of >70%, coverage >70%, and E-value < 1E-5 (Chen et al., 2005). To investigate the genes of putative antibiotic resistance, the ResFinder3.2 software was used to compare the assembled sequences with the antibiotic resistance gene sequence of reference in the ResFinder database (https://cge.cbs.dtu.dk/services/ResFinder/). For this analysis, the search parameters used were sequence identity >80% and coverage >60% (Zankari et al., 2012). The target antibiotics were as follows: aminoglycoside, beta-lactam, colistin, fosfomycin, fusidic acid, glycopeptides, macrolide, nitroimidazole, oxazolidinone, phenicol, quinolone, rifampicin, sulfonamide, tetracycline, and trimethoprim.
medium at 37°C overnight. The cells were harvested using centrifugation and suspended in phosphate-buffered saline to adjust the final cell concentration to 1.8 × 10^7 CFU/mL. Then, the cells were cultured in MRS medium at 37°C for 4 h before applying them to the API ZYM strips. After the reaction of the cells with the ZYM-A and ZYM-B reagents for 5 min, the color changes, which represent enzymatic activity, were determined using the color reaction chart.

### 2.7 Test for biogenic amine production

*L. lactis* IDCC 2301 was incubated in 10 mL of MRS medium at 37°C for 24 h. After centrifugation of the culture broth at 4000 rpm at 4°C for 30 min, the supernatant was obtained and filtered using a 0.22-µm pore size membrane. To extract the biogenic amines, 750 µL of the supernatant was mixed with the equivalent of 0.1 M HCL and filtered using a 0.45-µm membrane. Subsequently, 1 mL of the extracted biogenic amine was incubated in a water bath at 70°C for 10 min, followed by the addition of 20 µL of 2 M NaOH, 200 µL of saturated NaHCO₃, and 500 µL of dansyl chloride (10 mg/mL acetone) for derivatization. The derivatized biogenic amines were mixed with 200 µL of proline (100 mg/mL H₂O) and incubated in the dark at room temperature for 15 min. The mixture was made up to 5 mL with acetonitrile (high-performance liquid chromatography (HPLC) grade; Sigma-Aldrich). The derivatized biogenic amines were quantified using HPLC (LC-NETII/ADC, Jasco) with an Athena C18 column (4.6 × 250 mm, ANPEL Laboratory Tech.). The column was eluted with aqueous acetonitrile solution (67:33 of H₂O, v/v) at a flow rate of 0.8 mL/min. UV detector (UV-2075 plus, Jasco) at 254 nm was used to detect the biogenic amines, and quantification of the biogenic amines was performed using the calibration curves of each biogenic amine, including cadaverine, histamine, putrescine, 2-phenethylamine, and tyramine.

### 2.8 Measurement of lactate formation

To analyze lactate production by *L. lactis* IDCC 2301, the supernatants were obtained from *L. lactis* IDCC 2301 grown in 10 mL of MRS medium at 37°C for 24 h using centrifugation at 7000 rpm at 4°C for 30 min and filtration using a 0.22-µm pore size membrane. To quantify L- and D-lactate in the filtered supernatants, an assay kit (Megazyme) was used according to the manufacturers’ instructions. The absorbance at 340 nm of the reactant was measured using a microplate reader (Bio-Rad).

### 2.9 Acute oral toxicity of *L. lactis* IDCC 2301

Acute oral toxicity (AOT) test was carried out at the Korea Testing and Research Institute (KTR; Hwasun-gun). The AOT test was performed according to the guidelines of the organization for economic cooperation and development (OECD) for testing chemicals. In brief, female rats were randomly divided into four groups of three rats (two groups age 9 weeks; and others age 10 weeks) (OECD, 2008). Rats in the treatment group were orally administered 300 or 2000 mg of free-dried *L. lactis* IDCC 2301 powder in 10 mL sterilized water per kg body weight. Then, the mortality, signs of body weight changes, and toxicity were monitored for 14 days. On the 14th day, the rats were euthanized by injecting 100 mL isoflurane and an autopsy was conducted to examine the organs.

### 3 RESULTS

#### 3.1 Carbohydrate fermentation ability of *L. lactis* IDCC 2301

Lactic acid bacteria can grow on a variety of monosaccharides. Carbohydrates from 49 different sources were evaluated for fermentation patterns by *L. lactis* IDCC 2301, which is presented in Table 1. Based on the sources, different fermentation capabilities were observed. After incubation at 37°C for 48 h, among the 49, only 14 sources had been used as fermentation medium by the selected bacterial strain. However, ribose and monomeric carbohydrates, such as galactose, glucose, fructose, and mannose, were utilized efficiently compared with amygdaline, salicin, and amidon, whereas other sources had not been used at all.

#### 3.2 Determination of MIC and whole-genome analysis

*L. lactis* IDCC 2301 was assessed to determine whether it was susceptible to the nine antibiotics that are commonly used for the treatment of enterococcal infections (EFSA & FEEDAP, 2012; Ban et al., 2020). The nine antibiotics were ampicillin, clindamycin, chloramphenicol, erythromycin, gentamicin, kanamycin, streptomycin, tetracycline, and vancomycin. We determined the following MIC values: 64 µg/mL for vancomycin and tetracycline, 2 µg/mL for gentamicin, kanamycin, streptomycin, and vancomycin. The following MIC values: 2 µg/mL for chloramphenicol, 4 µg/mL for vancomycin and tetracycline, 2 µg/mL for ampicillin, and 1 µg/mL for erythromycin and clindamycin, indicating that *L. lactis* IDCC 2301 was susceptible to all the antibiotics tested (Table 2). The whole-genome sequence of *L. lactis* IDCC 2301 showed the highest similarity based on average nucleotide identity (ANI). The total length of the *L. lactis* IDCC 2301 genome was approximately 2.51 Mb with a GC content of 35.14% and 2403 functional genes (CDS) (Figure 1). In agreement with the MIC tests, the whole-genome analysis of *L. lactis* IDCC 2301 showed that it harbored no gene or similar genes associated with antibiotic resistance in its genome (Figure 1).

#### 3.3 Hemolytic property and biogenic amine production

To determine the pathogenicity of *L. lactis* IDCC 2301, an evaluation of hemolytic properties is tested. *L. lactis* IDCC 2301 was found
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TABLE 1 Carbohydrate utilization by \( Lactococcus lactis \) IDCC2301

| No. | Substrate       | Result | No. | Substrate       | Result | No. | Substrate       | Result | No. | Substrate       | Result |
|-----|-----------------|--------|-----|-----------------|--------|-----|-----------------|--------|-----|-----------------|--------|
| 1   | Glycerol        | −      | 14  | L-Sorbose       | −      | 27  | Cellobiose      | +      | 40  | D-Turanose      | −      |
| 2   | Erythritol      | −      | 15  | Rhamnose        | −      | 28  | Maltose         | +      | 41  | D-Lyxose        | −      |
| 3   | D-Arabinose     | −      | 16  | Dulcitol        | −      | 29  | Lactose         | −      | 42  | D-Tagatose       | −      |
| 4   | L-Arabinose     | −      | 17  | Inositol        | −      | 30  | Melibiose       | −      | 43  | D-Fucose         | −      |
| 5   | Ribose          | +      | 18  | Mannitol        | −      | 31  | Sucrose         | +      | 44  | L-Fucose         | −      |
| 6   | D-Xylose        | −      | 19  | Sorbitol        | −      | 32  | Trehalose       | +      | 45  | D-Arabitol       | −      |
| 7   | L-Xylose        | −      | 20  | \( \alpha \)-Methyl-D-mannoside | − | 33  | Inulin         | −      | 46  | L-Arabitol       | −      |
| 8   | Adonitol        | −      | 21  | \( \alpha \)-Methyl-D-glucoside | − | 34  | Melezitose      | −      | 47  | Gluconate        | −      |
| 9   | \( \beta \)-Methyl-xylose | − | 22  | N-Acetyl-glucosamine | + | 35  | D-Raffinose     | −      | 48  | 2-keto-gluconate | −      |
| 10  | Galactose       | +      | 23  | Amygdaline      | +\( w \) | 36  | Amidon         | +\( w \) | 49  | 5-keto-gluconate | −      |
| 11  | D-Glucose       | +      | 24  | Arbutine        | −      | 37  | Glycogen        | −      |     |                 |        |
| 12  | D-Fructose      | +      | 25  | Esculin         | +      | 38  | Xylitol         | −      |     |                 |        |
| 13  | D-Mannose       | +      | 26  | Salicin         | +\( w \) | 39  | Gentibiose      | +      |     |                 |        |

\((+), \text{utilization}; (+w), \text{weak utilization}; (−) \text{non-utilization}.)

TABLE 2 Minimum inhibitory concentration (MIC) of \( Lactococcus lactis \) IDCC 2301 against nine antibiotics

| Cutoff value\(^a\) (μg/mL) | AMP  | CLI  | CHL  | ERY  | GEN  | KAN  | STR  | TET  | VAN  |
|-----------------------------|------|------|------|------|------|------|------|------|------|
| 2                           | 1    | 8    | <0.125/S | 32   | 64   | 32   | 4    | 4    |      |
| L. lactis IDCC 2301         | 0.25/S | 0.125/S | 4/S  | <0.125/S | 8/S  | 16/S | 16/S | 0.25/S | 1/S  |

\(^a\)EFSA (European Food Safety Authority) (EFSA & FEEDAP, 2012).

3.4 | Enzymatic activity test

Since enzymatic activity of microbes is an important factor for safety of probiotics, we tested various enzymatic activities including \( \beta \)-glucuronidase, esterase, and lipase. In our study, \( L. lactis \) IDCC 2301 found to have no \( \beta \)-glucuronidase activity (Table 4). However, five enzymes showed positive activities, namely, leucine arylamidase, \( \alpha \)-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, and \( \beta \)-glucosidase. However, most of the enzymes did not show any activity. Thus, \( L. lactis \) IDCC 2301 seems not to produce toxic chemicals during the fermentation.

3.5 | Lactate formation of \( L. lactis \) IDCC 2301

Lactic acid bacteria can produce lactate from pyruvate using homofermentative or hetero-fermentative methods (Drinan et al., 1976). Based on the strains and growth conditions, L-lactate is predominantly produced; however, D-lactate can also be produced from pyruvate (Zuniga et al., 1993). Because it is difficult for D-lactate to be metabolized, it can accumulate in the human body, leading to D-lactic acidosis (Petersen, 2005; Schiraldi et al., 2003). Thus, D-lactate formation by LAB is a critical factor for safety assessment. In this study, the production of lactate by \( L. lactis \) IDCC 2301 was verified to be 100% of L-lactate (Table 5).

3.6 | Single-dose acute oral toxicity of \( L. lactis \) IDCC 2301

To evaluate the safety of \( L. lactis \) IDCC 2301 in vivo, single-dose acute oral toxicity tests with four test groups were conducted for 14 days (Table 6). It was observed that a single oral dose of \( 0.23 - 1.6 \times 10^{12} \) CFU and \( 0.22 - 1.6 \times 10^{12} \) CFU of \( L. lactis \) IDCC 2301 did not cause mortality and toxicity in 9-week-old and 10-week-old rats,
respectively. Moreover, there were no significant changes in the appearances, behaviors, body weight (Table 6), and feed intake of the rats. Throughout the autopsy, no gross pathological change was observed in all rats. Therefore, it was concluded that there is no evidence of any toxicity caused by \textit{L. lactis} IDCC 2301 in the rats.

4 | DISCUSSION

Studies have shown that \textit{L. lactis} have immense bioeconomic value because of its diverse applications in food industries. Although \textit{L. lactis} is considered a nonpathogenic organism, care must be taken before it is used for human consumption. Recently, Karaaslan \textit{et al.}, Topçu \textit{et al.}, and Uchida \textit{et al.} reported symptoms of \textit{L. lactis} infection in children (Karaaslan \textit{et al.}, 2016; Topçu \textit{et al.} 2011; Uchida \textit{et al.}, 2011). Hence, efficient use of lactic acid bacteria requires a complete understanding of many aspects of bacterial physiology, such as consumption of proteins and sugars for growth, lactate formation from sugars, synthesis of flavoring substances involved in cheese, and relation in different fermentations. In this study, we investigated the safety properties of \textit{L. lactis} IDCC 2301 isolated from homemade cheese using in vitro assays, and these bacteria
demonstrated susceptibility against antibiotics, enzymatic activity, and L-lactate formation. The safety assessment results revealed that there is no evidence of toxicity caused by *L. lactis* IDCC 2301. *L. lactis* strains are considered food-grade organisms, and they are used in a variety of food and feed preparations.

For further characterization, the *L. lactis* strain was grown at 37°C for 48 h and the carbohydrate consumption patterns were monitored.

**TABLE 4** Enzymatic activities of *L. lactis* IDCC2301

| Enzyme            | Activity |
|-------------------|----------|
| Alkaline phosphate| −        |
| Esterase          | −        |
| Esterase lipase   | −        |
| Lipase            | −        |
| Leucine arylamidase| +      |
| Valine arylamidase| −        |
| Cysteine arylamidase| −      |
| Trypsin           | −        |
| α-Chymotrypsin    | +        |
| Acid phosphatase  | +        |
| Naphthol-AS-Bi-phosphohydrolase| + |
| α-Galactosidase   | −        |
| β-Galactosidase   | −        |
| β-Glucuronidase   | −        |
| α-Glucosidase     | −        |
| β-Glucosidase     | +        |
| N-Acetyl-β-glucosaminidase| −   |
| α-Mannosidase     | −        |
| α-Fucosidase      | −        |

**TABLE 5** The ratio of L- to D-lactate produced by *L. lactis* IDCC2301

| Strain            | L-Lactate (mg/mL) | D-Lactate (mg/mL) | Ratio (%) |
|-------------------|------------------|------------------|-----------|
| *L. lactis* IDCC2301 | 18.27 ± 0.40     | N.D.             | 100       |

Abbreviation: N.D., not detected

**TABLE 6** Body weight changes in the tested rats

| Dose (mg/kg B. W.)^a| Group         | Day(s) after administration | 0      | 1      | 3      | 7      | 14     | 14     |
|---------------------|---------------|-----------------------------|--------|--------|--------|--------|--------|--------|
|                     |               |                             |        |        |        |        |        |        |
| Li\(^b\), 300       | 9-week-old    |                             | 215.2  ± 4.7 | 236.4 ± 9.1 | 243.9 ± 5.7 | 256.5 ± 9.5 | 260.5 ± 7.0 |        |
|                     | 10-week-old   |                             | 229.4  ± 1.0 | 253.2 ± 2.2 | 255.8 ± 3.3 | 264.0 ± 2.7 | 267.7 ± 4.8 |        |
| Li, 2000            | 9-week-old    |                             | 216.5  ± 15.1 | 237.4 ± 20.7 | 242.1 ± 17.5 | 254.1 ± 22.4 | 264.8 ± 24.5 |        |
|                     | 10-week-old   |                             | 231.6  ± 11.6 | 250.0 ± 11.5 | 257.9 ± 9.3  | 273.3 ± 9.3  | 280.4 ± 13.7 |        |

Abbreviation: BW, body weight.

^a Li, Lactococcus lactis IDCC 2301.
common commensal microorganisms. Observation of physical changes by hemolytic activity after culturing the selected microorganism on an animal or human blood-containing medium is commonly used to evaluate the hemolytic activity. In this study, L. lactis IDCC 2301 showed no hemolytic activity on sheep blood agar plates.

Biogenic amines, decarboxylation of amino acid products, and low molecular weight compounds are produced by lactic acid bacterial fermentation (Barbieri et al., 2019). These compounds have some negative health effects when the amine-metabolizing power of the human body deteriorates (Alvarez & Moreno-Arribas, 2014). These amines can be formed in fermented food during fermentation or decarboxylase activity by bacteria. However, the type and level of biogenic amines formed in food products depend on the composition of the food and the growth of bacteria during the processing and storage of foods. Protein-rich fermented foods contain a high amount of biogenic amines (Jeong & Lee, 2015). For food safety purposes, tyramine and histamine are considered the most important amino acids and are responsible for various poisoning in fish and food-induced migraines (Ban et al., 2020). In our study, biogenic amines, including tyramine, histamine, putrescine, 2-phenethylamine, and cadaverine, were not detected in L. lactis IDCC 2301.

From the enzymatic activity perspective, the screened L. lactis strain was determined using the semi-quantitative API ZYM system. β-Glucosidase of lactic acid bacteria can hydrolyze glucose conjugates from plants, forming a variety of secondary metabolites. These metabolites function as health-promoting substances (e.g., antioxidants) (Michlmayr et al., 2013). However, the enzymatic activity of β-glucuronidase is important; it can indicate whether the carcinogenic compounds are present or not. It has been reported that β-glucuronidase produced by microorganisms can develop toxic steroids, such as estrogen, or carcinogenic compounds, resulting in an increased risk for colorectal cancer (Kim & Jin, 2001). In this study, we confirmed L. lactis IDCC 2301 has no β-glucuronidase activity. The leucine arylamidase activity of the selected strain of L. lactis was similar to that reported by Requena et al. and Otero et al. (Otero et al., 2001; Requena et al., 1991). However, the activities of valine and cystine arylamidase were not detected. These results are consistent with those of several studies (Otero et al., 2001; Requena et al., 1991). Tzanetakis & Litopoulou-Tzanetaki, 1989). Requena et al. and Otero et al. reported similar results and found that the activity of leucine arylamidase was high in some strains of L. plantarum and L. casei subsp. casei (Otero et al., 2001; Requena et al., 1991). Considering all these results, L. lactis IDCC 2301 could be suitable probiotics for industrial application with respect to safety issues.

5 | CONCLUSION

The utilization of probiotics in the food industry is gaining popularity, especially in the dairy sector. However, safety evaluation is the most important criterion before using the selected probiotic for human consumption. For safety assessment, antibiotic susceptibility, biogenic amine formation, and hemolytic activities need to be considered according to the 2002 FAO/WHO guideline. In this study, we evaluated the safety parameters of L. lactis IDCC 2301 isolated from homemade cheese. However, we did not find any antibiotic-resistant gene through antibiotic susceptibility testing. Moreover, no biogenic compounds were produced after 24 h of incubation with the formation of only L-lactate (18.27 ± 0.40 mg/mL). Poor hemolytic activity is considered a low risk for humans, and fortunately, in our study, the strain showed negative activity on sheep blood agar. Depending on the antibiotic, MIC values ranged from 1 (erythromycin and clindamycin) to 64 (kanamycin). L. lactis 2301 showed some enzymatic activities, but, importantly, β-glucuronidase activity was absent. Hence, L. lactis IDCC 2301 can be used safely as a starter organism in cheese and other fermentation processes because they did not produce biogenic amines and did not show any hemolytic activity.

CONFLICTS OF INTEREST

All authors declare that they have no competing interests.

ETHICS APPROVAL

Not applicable.

CONSENT FOR PUBLICATION

All authors read and approved the final manuscript for publication.

DATA AVAILABILITY STATEMENT

Data are openly available in a public repository that issues datasets with DOIs.

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