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Safety assessment of *Tetragenococcus halophilus* isolates from doenjang, a Korean high-salt-fermented soybean paste

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ABSTRACT

We assessed the safety of 49 *Tetragenococcus halophilus* strains isolated from doenjang in Korea. Minimum inhibitory concentration assays showed that all strains can be considered as susceptible to ampicillin, erythromycin, penicillin G, tetracycline, and vancomycin, but resistant to ciprofloxacin based on the *Enterococcus* breakpoint values provided by the European Committee on Antimicrobial Susceptibility testing in 2015. Ciprofloxacin resistance was sufficiently high to consider the potential for acquisition of transmissible determinants. Two strains exhibiting potentially acquired resistance to chloramphenicol and gentamicin, and chloramphenicol alone, were identified. None of the strains exhibited α-hemolytic activity or biofilm formation; two strains exhibited weak β-hemolytic activity. Doenjang isolates produced an average of 3338.6 ppm of tyramine in the laboratory, considerably higher than the levels produced by two reference strains. All of the test strains exhibited similar cadaverine, histamine, and putrescine production patterns. Most *T. halophilus* strains could grow at a NaCl concentration >18%, exhibited acid production at 15% NaCl, and expressed strain-specific protease and lipase activities. The potential acquisition of transmissible determinants for antibiotic resistance and tyramine production identified in this study necessitate the need for a thorough safety assessment of *T. halophilus* before it can be considered for use in food fermentation processes.

1. Introduction

Tetragenococcus halophilus, formerly classified as *Pediococcus halophilus*, is a Gram-positive, tetrad-forming, nonmotile, catalase-negative, and halophilic lactic acid bacterium that produces acid from glucose without gas formation. *T. halophilus* has been widely detected in high-salt-fermented food products including fermented fish products, soy pastes, and soy sauce, and is considered a potential starter for their production (Devi et al., 2015; Hanagata et al., 2003; Juste et al., 2008; Kim et al., 2010; Onda et al., 2002; Roling and van Berseveld, 1996; Udomsil et al., 2010).

Udomsil et al. (2011) showed that *T. halophilus* inoculation could enhance the flavor of fish sauce through the production of volatile compounds. The effectiveness of *T. halophilus* at improving the flavor of soy sauce was proven by the co-culture of two yeasts, *Zygosaccharomyces rouxii* and *Candida versatilis* (Cui et al., 2014). *T. halophilus* also contributed to reduce the browning pigments, which is one of the problems associated with long-time incubation of fermented soy sauce, through the elimination of pentose (Abe and Uchida, 1989). *T. halophilus* strain Th221 isolated from the soy sauce fermentation process was reported to possess immunomodulatory activity, promoting T helper type 1 immunity (Masuda et al., 2008) and the efficacy of perennial allergic rhinitis (Nishimura et al., 2009). These results indicated the potential of this species as a starter to improve the sensory properties as well as the health functionality of fermented foods.

Fermented foods have a long history of human consumption, thus the predominant microbes, mainly lactic acid bacteria (LAB), involved in fermentation have generally been considered safe. However, increasing reports of their association with serious infections, including bacteremia, endocarditis, and localized abscesses (Adams, 1999) has meant that the organisms are no longer automatically considered safe. A number of studies have been performed to assess the safety of species of the genera *Enterococcus*, *Lactobacillus*, *Lactococcus*, and *Leuconostoc* from fermented foods (Ammor et al., 2007; Fraqueza, 2015; Jeong and Lee, 2015; Jeong et al., 2015; Perin et al., 2014). However, no safety assessments

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have been reported for the genus *Tetragenococcus*. Therefore, although several LAB featured in the Qualified Presumption of Safety (QPS) list published by the European Food Safety Authority (EFSA), *T. halophilus* did not qualify for the latest edition (EFSA, 2015).

Recently, we identified *T. halophilus* as the predominant species present in doenjang, a traditional Korean high-salt-fermented soybean paste that is ripened at a concentration of >12% (w/w) NaCl (Jeong et al., 2014b). Its dominance in doenjang suggests its potential as a starter culture for the mass production of this product. Therefore, in this study, we assessed the antibiotic susceptibility, hemolytic activity, biofilm formation, and biogenic amine production of 49 *T. halophilus* strains isolated from doenjang (Jeong et al., 2014b) to assess the safety of Korean isolates according to the EFSA guidelines for the safe use of microorganisms as food/feed materials (EFSA, 2004). The salt tolerance, protease and lipase activities, and acid production of *T. halophilus* isolates were also characterized to select possible starter candidates for quality doenjang manufacture.

2. Materials and methods

2.1. Bacterial strains and culture conditions

A total of 52 *T. halophilus* strains were used in the current study. Forty-nine strains were previously isolated from two doenjang samples (Jeong et al., 2014b) and the type strain KCCM 40909^T and two published strains KCCM 40823 and KCCM 11377 were purchased from the Korean Culture Center of Microorganisms (Seoul, Korea). Bacterial strains were cultured in de Man–Rogosa–Sharpe (MRS) medium (Difco, Detroit, MI, USA) containing 3% (w/v) NaCl at 30 °C for 48 h under semi-anaerobic conditions using tightly capped tubes fully filled with media.

2.2. Determination of minimum inhibitory concentrations (MICs)

Antibiotic MICs were determined by the broth microdilution method according to the guidelines of the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2007). Eight antibiotics (ampicillin, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, penicillin G, tetracycline, and vancomycin) were selected among those frequently used to test the antibiotic susceptibility of LAB (Perez-Pulido et al., 2006; Perin et al., 2014; Pinheiro et al., 2004). A 2-fold serial dilution was prepared for each antibiotic in deionized water, and final concentrations in each well of the microplate ranged between 0.5 and 512 mg/L. *T. halophilus* strains were cultured twice in MRS broth containing 3% NaCl and matched to a McFarland 0.5 turbidity standard (bioMerieux, Marcy l'Etoile, France). The cultured strains were further diluted (1:100) in cation-adjusted Mueller–Hinton broth (Oxoid, Basingstoke, UK) containing 3% NaCl and 2% (v/v) horse blood (KisanBio, Seoul, Korea) to achieve the desired inoculum concentration. The final inoculum density in each well was 5×10^5 colony forming units/mL. Microplates were then incubated at 35 °C for 20 h under static conditions. The MIC of each antibiotic was recorded as the lowest concentration at which no turbidity was observed in the wells. Resistance to a particular antibiotic was defined as the point at which the MIC value of a tested antibiotic was higher than the recommended breakpoint value of *Enterococcus faecium* and *Enterococcus faecalis* as defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST; http://mic.eucast.org) in 2015. All of the experiments were conducted five times on separate days.

2.3. Hemolytic activity test

MRS agar supplemented with 5% (v/v) rabbit blood (MB Cell, Seoul, Korea) or 5% (v/v) sheep blood (BBL Microbiology Systems, Sparks, MD, USA) was used for α- and β-hemolytic activity tests, respectively. Alpha-hemolytic activity was determined by incubation at 30 °C for 48 h, while β-hemolytic activity was determined by cold shock at 4 °C for 24 h after incubation at 30 °C for 48 h, as described previously (Jeong et al., 2014a) with minor modifications. Hemolytic activities were determined by the formation of clear lytic zones around colonies on each blood-containing MRS agar plate. *Staphylococcus aureus* USA300-P23 was used as a positive control for hemolytic analyses. Three independent experiments were conducted.

2.4. Biofilm formation assay

An overnight culture in MRS broth supplemented with 3% NaCl was diluted 200-fold with the same fresh medium. A 200-μL aliquot of culture was then added to each well of a 96-well microtiter plate and incubated for 48 h at 30 °C under static conditions. After the medium was discarded, the plate was dried, and the cells were stained with 0.1% (w/v) safranin (Heilmann et al., 1996). A positive safranin staining result indicated biofilm formation. *Staphylococcus equorum* DSM 20674^T was used as a positive control for biofilm formation analysis (Jeong et al., 2013). Three independent experiments were conducted.

2.5. Analysis of biogenic amine production

Stationary phase cell cultures were normalized by measuring the optical density at 600 nm, and then 1:100 dilutions of the normalized cell cultures were inoculated into MRS broth containing 3% NaCl and four biogenic amine precursors. The precursors, L-tyrosine disodium salt hydrate, L-histidine monohydrochloride monohydrate, L-lysine monohydrochloride, and L-ornithine monohydrochloride (pH 5.8), were added to a final concentration of 0.25% (w/v), and pyridoxal-HCl was added to a final concentration of 0.0005% (w/v) (Sigma, St Louis, MO, USA) (Bover-Cid and Holzapfel, 1999). Cultures were incubated for 3 days at 30 °C under semi-anaerobic conditions, and then 2-mL aliquots of the culture broths were dansylated with dansyl chloride according to a previously described method (Hwang et al., 1997) with minor modifications. The dansyl derivatives of the biogenic amines were dissolved in 5 mL of acetonitrile, and 10-μL aliquots were used for high performance liquid chromatography. Calibration curves for quantification were constructed using each authentic compound.

The biogenic amines produced by strains were determined using an Agilent Technologies HPLC 1200 series system (Palo Alto, CA, USA) monitored by a UV detector at 254 nm, and a Nova-Pak C18 column (4 μm, 150 × 4.6 mm, Waters, Milford, MA, USA) was used for chromatographic separation. The gradient elution program was initiated with 50:50 (v/v) acetonitrile:0.1 M ammonium acetate at a flow rate of 1 mL/min, followed by a linear increase to 90:10 acetonitrile:0.1 M ammonium acetate for 19 min, and was then decreased to 50:50 over the final 2 min. All of the experiments were conducted three times on independent samples prepared in the same way on separate days.

2.6. Amplification of the histidine decarboxylase and tyrosine decarboxylase genes

The genomic DNA of *T. halophilus* strains was extracted using a DNeasy Tissue Kit (Qiagen, Hilden, Germany). The polymerase chain reaction (PCR) amplification of histidine decarboxylase and