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Safety and technological characterization of *Staphylococcus equorum* isolates from jeotgal, a Korean high-salt-fermented seafood, for starter development

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ABSTRACT

To select starters for jeotgal, a traditional Korean high-salt-fermented seafood, the safety and technological properties of its predominant bacteria isolates, which were identified as *Staphylococcus equorum*, were assessed. Among the 185 *S. equorum* isolates from jeotgal, 126 ampicillin-sensitive strains were subjected to assessments for antibiotic susceptibility and safety hazards. Sixty-six out of the 126 *S. equorum* strains exhibited phenotypic resistances to at least one antibiotic, and their prevailing resistances were to penicillin G (34.1%), erythromycin (9.5%) and trimethoprim (9.5%). Twenty-four *S. equorum* strains expressed resistance to at least two antibiotics. The *lnuA* for lincomycin (four strains) and *pbp* for β-lactam (three strains) were amplified by PCR. α-Hemolytic activity was not detected from the 126 strains, and 87 strains presented δ-hemolytic activity. Among the 87 strains, three strains exhibited β-hemolytic activity. Thirty-seven strains formed a biofilm. A hemolysin gene homologous to that of *Staphylococcus epidermidis* was amplified from an *S. equorum* strain with β-hemolytic activity by PCR; however, no PCR product homologous to the previously known staphylococcal enterotoxin genes was amplified. Thirty-nine *S. equorum* strains cleared all of the tested safety hazards and were adopted for technological property assessments. Among these strains, 16 strains exhibited protease, lipase and nitrate reductase activities, and seven strains did not produce four types of biogenic amines. Five biogenic amine non-producers exhibited three enzyme activities. Most of the strains could grow on the agar with 20% NaCl, and 13 strains maintained growth at the 25% NaCl condition. *S. equorum* KS1039, which is the most applicable strain that covers the safety and technological requirements for jeotgal, can grow at the 25% NaCl condition. Through this research study, we reconfirmed the necessity of characterization in the functionality and safety of *S. equorum* for starter development because all of the tested phenotypic characteristics were expressed in strain-specific manners.

1. Introduction

Jeotgal, a Korean high-salt-fermented seafood, is made by adding 20 to 30% (w/w) salt to various types of seafood, such as shrimp, oyster, shellfish, fish, fish eggs, and fish intestines, and becomes palatable through subsequent ripening and fermentation. Jeotgal has been known to attain rich flavors and specific structures through autolytic and microbial proteolysis during the preservation period (Jung et al., 2013). Typically, jeotgal requires a long fermentation period of more than six months to yield qualified products. The qualities of the products are dependent on the environmental conditions, and sometimes, inconsistent final products are obtained due to uncontrolled fermentation (Hur, 1996; Lee, 1989). Starter culture application is a common practice for the quality assurance of fermented foods and may be a promising solution to accelerate the ripening speed and to assure the quality of jeotgal.

We recently analyzed the cultivable bacterial communities in two types of the most commonly consumed jeotgal to develop a more complete overview of the bacterial community in jeotgal and to determine the predominant species (Guan et al., 2011). The predominant species will be possible starter candidates for jeotgal fermentation to assure jeotgal quality and to accelerate the fermentation time. Twenty-five strains and 190 strains of coagulase-negative staphylococci (CNS) were isolated from Myeolchi-jeotgal made with anchovy (*Engraulis japonicus*) and Saeu-jeotgal made with tiny shrimp (*Acetes japonicus*), respectively. CNS comprised 40% of the total isolates in Saeu-jeotgal, and *Staphylococcus equorum* was the most populous species.

CNS have been isolated from various high-salt-fermented foods (Blaiotta et al., 2004; Cocolin et al., 2001; Corbiere Morot-Bizot et al., 2006; Drosinos et al., 2007) and are known to play a major role in the development of sensory properties by the reduction of nitrates to nitrite and then to nitrous oxide, as well as through proteolysis and lipolysis (Hammes and Hertel, 1998). In particular, *Staphylococcus carnosus*, *Staphylococcus xylosus* and *S. equorum* were reported to produce low-molecular-weight compounds, including esters, amino acids, aldehydes, amines and free fatty acids, which have an impact on flavor (Berdague et al., 1993; Sondergaard and Stahnke, 2002; Stahnke, 1994). Although CNS have been reported as the main species in fermented sausages and meats (Irlinger, 2008; Novakova et al., 2006), some CNS have been reported to cause bloodstream infections in extremely-low-birth-

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weight babies and have a virulence factor for biofilm formation (Cheung and Otto, 2010; Longauerova, 2006; von Eiff et al., 2002). Therefore, the safety assessment of starter candidates is the prerequisite for the future application of CNS in the jeotgal manufacturing process.

The safety of microbial food additives, such as starters, is mainly assessed by two approaches: the Generally Recognized as Safe notification program of the US Food and Drug Administration and the Qualified Presumption of Safety (QPS) system of the European Food Safety Authority (EFSA) (EFSA, 2004; Wessels et al., 2004). Recently identified important issues of these systems are the presence of acquired antibiotic resistance in food bacteria and the production of toxin and biogenic amines in final products (EFSA, 2009; Naila et al., 2010). Therefore, several studies have reported the safety of microbes from various fermented foods, such as fermented meats, dairy products and soy sources (Clementi and Aquilanti, 2011; Marty et al., 2012; Stute et al., 2002; Talon and Leroy, 2011).

Several types of traditional fermented foods are consumed in Korea: kimchi, fermented vegetables; doenjang, fermented soybean foods, and jeotgal. The demand for commercial traditional fermented foods is increasing due to economic growth, changes in the family structure, development of the food processing industry, and increasing number of women working outside the home. The application of a starter culture is an option for obtaining qualified products by mass production. However, the safety assessment of the microbial additives has rarely been mentioned in studies concerning starter development. A recent study that assessed the antibiotic resistance and biogenic amine production of lactic acid bacteria isolated from kimchi is the only report that has described a safety assessment (Lee et al., 2011). In this study, we assessed the safety- and function-related properties of *S. equorum* isolates from jeotgal to select safe starter candidates.

2. Materials and methods

2.1. Bacterial strains and culture conditions

In this study, 185 *S. equorum* strains isolated and identified in our previous studies were used for safety assessment and technological characterization (Guan et al., 2011; Jeong et al., 2013). The bacterial strains were mainly cultured in tryptic soy agar (TSA) and tryptic soy broth (TSB) at 30 °C for 24 h to maintain their traits. The salt tolerances of the strains were determined by assessing growth on TSA supplemented with up to 25% (w/v) NaCl at 30 °C for 72 h.

2.2. Antibiotic susceptibility test

Strains cultured on TSA were transferred to the same media containing ampicillin (32 μg/ml) by tooth-picking and incubated at 30 °C for 2 days (Boothe, 2006). The ampicillin-sensitive strains, which did not grow on TSA containing ampicillin, were selected for further antibiotic susceptibility testing using the disk diffusion method on Mueller–Hinton agar at 30 °C for 24 h. The guidelines for CNS of the Clinical and Laboratory Standards Institute (http://www.clsi.org/) and of the European Committee on Antimicrobial Susceptibility Testing (http://www.eucast.org) were applied. Fourteen types of antibiotic disks containing amikacin (30 μg), cefoxitin (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), erythromycin (15 μg), gentamicin (10 μg), lincomycin (15 μg), linezolid (10 μg), ofloxacin (5 μg), oxacillin (1 μg), penicillin G (10 units), rifampicin (5 μg), tetracycline (30 μg), and trimethoprim (5 μg) were purchased from Oxoid (Basingstoke, Hants, UK).

2.3. PCR for the identification of antibiotic resistance and virulence determinants

The genomic DNA of *S. equorum* strains was extracted using a DNeasy Tissue Kit (Qiagen, Hilden, Germany). The amplification of antibiotic resistance, hemolysin, and enterotoxin genes was performed with the specific primer sets shown in Tables 1 and 2 using a T-3000 thermocycler (Biometra, Gottingen, Germany). The primer sets that were successfully applied to amplify the antibiotic resistance and virulence determinants from Gram-positive bacteria, mainly staphylococci, were adopted. The primers for *aphA*, *cat*, *cfr*, *pbp*, *norA*, *dfrA*, and *hlb* genes were designed from the known DNA sequences of *Staphylococcus* species. The PCR mixture consisted of the template DNA, 0.5 μM of each primer, 1.25 units of Inclone™ Taq polymerase (Inclone Biotech, Korea), 100 mM dNTPs, and 2.5 mM $MgCl_2$. The samples were preheated for 5 min at 95 °C and then amplified using 30 cycles of 1 min at 95 °C, 30 s at 57 °C and 1 min at 72 °C. The amplified PCR products were sequenced using a custom service provided by GenoTech (Korea). The web-hosted BLAST program was used to find the sequence homologies of the amplified fragments with known gene sequences in the National Center for Biotechnology Information database.

2.4. Hemolytic activity test

The α-hemolytic activity was determined on TSA containing 5% horse blood (HB agar, KisanBio, Korea) through clear halo formation after incubation at 37 °C for 24 h. TSA supplemented with 5% sheep blood (SB agar, BBL Microbiology Systems, USA) was used for β- and δ-hemolytic activity tests. The β-hemolytic activity was determined by incubation at 37 °C for 24 h followed by cold shock at 4 °C for 12 h because β-toxin interacts with sheep red blood cells but does not lyse the cells at 37 °C. The δ-hemolysin is known to result in synergistic and complete hemolysis with β-hemolysin-producing staphylococci (Hebert and Hancock, 1985). Therefore, the δ-hemolytic activity was determined by cross-streaking the test strains perpendicularly to *Staphylococcus aureus* RN4220, which produces only β-hemolysin (Traber and Novick, 2006). The δ-hemolytic activity was denoted by an enhanced area of hemolysis at the intersection of *S. aureus* RN4220 and test strain streaks. *S. aureus* Newman (Duthie and Lorenz, 1952) and *S. aureus* USA300-P23 (Jeong et al., 2011) were used as controls for the hemolytic analysis. All of the experiments were conducted at least three times on separate days.

2.5. Biofilm formation assay

An overnight culture in TSB was diluted 200-fold with fresh TSB containing 0.5% glucose. In total, 200 μl of culture was added to 96-well microtiter plates and incubated for 24 h at 37 °C without shaking. After the supernatant was discarded, the plates were dried, and the cells were stained with 0.1% safranin (Heilmann et al., 1996).

2.6. Enzyme activity determination

The protease and lipase activities of the isolates were determined on agar media containing 2% skim milk (w/v) and 1% tributyrin (v/v, Sigma, USA), respectively. Skim milk was added to TSA, whereas tributyrin was added to tributyrin agar (Sigma) and emulsified by sonication before autoclaving. Colonies cultured on TSA were transferred to each substrate-supplemented agar medium and incubated at 30 °C for 48 h. The size of the clear zone around the colony was used as the indicator of enzyme activity.

The nitrate reductase activity was determined as previously described (Miralles et al., 1996) with minor modifications. Colonies cultured on TSA were transferred to YT (1.0% tryptone, 0.5% yeast extract, pH 7.0) agar supplemented with 0.1% KNO_3. After incubation at 30 °C for 20 h, the plates were flooded with a mixture composed of equal amounts of solution NIT1 (0.8 g of sulfanilic acid in 100 ml of 5 N acetic acid) and NIT2 (0.6 g of *N,N*-dimethyl-1-naphthylamine in 100 ml of 5 N acetic acid) for the detection of nitrite. The appearance of red haloes around colonies indicates the presence of nitrate reductase activity.