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Safety and technological characterization of coagulase-negative staphylococci isolates from traditional Korean fermented soybean foods for starter development

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ABSTRACT

To select starters for the production of meju and doenjang, traditional Korean fermented soybean foods, we assessed the safety and technological properties of their predominant isolates, *Staphylococcus saprophyticus*, *Staphylococcus succinus* and *Staphylococcus xylosus*. Phenotypic antibiotic resistance, hemolysis and biofilm formation were strain-specific. None of the *S. succinus* isolates exhibited antibiotic resistance or hemolytic activities. Thirty-three selected strains, identified through safety assessments of 81 coagulase-negative staphylococci (CNS) isolates, produced cadaverine, putrescine, and tyramine, but not histamine in the laboratory setting. The production of these three biogenic amines may, however, be insignificant considering the high levels of tyramine produced by the control, *Enterococcus faecalis*. The 33 CNS strains could grow on tryptic soy agar containing 21% NaCl (w/v), exhibited acid producing activity at 15% NaCl, and expressed strain-specific protease and lipase activities. *S. succinus* 14BME1, the selected starter candidate, produced significant amounts of benzeneacetic acid, 2,3-butanediol, trimethylpyrazine, and tetramethylpyrazine through soybean fermentation.

1. Introduction

Coagulase-negative staphylococci (CNS) are found on skin and mucous membranes of mammals, and are ubiquitously distributed in a vast array of natural niches, including soil, water and air, and in a variety of foodstuffs, such as meat, cheese and raw milk (Coton et al., 2010; Götz et al., 2006; Irlinger, 2008). CNS are generally described as benign bacteria, contrary to the coagulase-producing *Staphylococcus aureus*, and infection by food-related CNS is rare. *Staphylococcus carnosus*, *Staphylococcus equorum*, *Staphylococcus succinus*, and *Staphylococcus xylosus* represent a significant proportion of the intrinsic bacteria found in naturally fermented meat products, and have been used as starter cultures in meat fermentation processes (Seitter et al., 2011; Talon et al., 2008). They enhance flavor development through proteolysis and lipolysis, and improve color by reducing nitrates to nitrite, and thereafter to nitrous oxide (Hammes and Hertel, 1998). Meanwhile, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, and *Staphylococcus saprophyticus* have been known to lead to the occasional opportunistic infections (Kacica et al., 1994; Natoli et al., 2009; Widerstrom et al., 2012).

Recently, CNS have been identified as a dominant group of bacteria in traditional Korean fermented foods (Guan et al., 2011; Jeong et al., 2014b; Jung et al., 2013, 2014; Nam et al., 2012). In our studies on jeotgal, a Korean high-salt-fermented seafood, CNS were the predominant type of bacteria (Guan et al., 2011; Jeong et al., 2014a). We also found that CNS constituted a significant proportion of the microflora in meju and doenjang (25.6% of the total number of isolates) (Jeong et al., 2014b). Doenjang, a traditional Korean fermented soybean paste, is made by mixing and ripening meju with a high salt brine. Meju, a naturally fermented soybean product, is prepared by soaking, steaming, crushing, and molding the soybean into blocks and allowing it to ripen for 1–2 months. The ripened meju is a source of naturally occurring microorganisms and enzymes that degrade macromolecules in the soybean block. Meju supplies nutrients, flavors, enzymes, and microorganisms not only in the production of doenjang but also other traditional Korean fermented seasonings including ganjang (soy sauce) and gochujang (hot pepper paste).

In the typical production of meju and doenjang, no control measures are implemented, which sometimes results in inconsistent product quality, thereby hindering the commercialization of both products. Starter culture application is a common practice for quality assurance of fermented foods and it offers a promising solution for accelerating the ripening speed of the food, as well as standardizing the quality and reducing the health safety hazards associated with fermented foods (Leroy and De Vuyst, 2004; Leroy et al., 2006). Recently, several starter culture candidates have been selected to produce quality

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fermented soybean products and for reducing the likelihood of consuming fermented foods containing hazardous microorganisms (Hong et al., 2016; Lee et al., 2014; Mo et al., 2010; Shukla et al., 2014, 2015). Food pathogen *Bacillus cereus* as well as the aflatoxins and biogenic amines produced by naturally occurring microorganisms are known to be major safety hazards in Korean fermented soybean foods. The selected starters have been reported to fulfill their designated roles in producing fermented soybean foods. *Bacillus* species are acknowledged to be the primary candidate in such foods because they have been detected as the predominant bacteria in Korean fermented soybean foods and are generally considered to be safe microorganisms in food.

Meanwhile, CNS strains isolated from Korean fermented foods can grow on a medium containing up to 25% NaCl (w/v), and exhibit protease and lipase activities, which suggests that they may play important roles in the manufacturing of high-salt Korean fermented foods (Jeong et al., 2014a, 2014b, 2016; Jeong and Lee, 2015). However, the lack of safety data as well as the history of CNS use in food fermentation in Korea hinder their introduction for use in Korean food fermentation. Proving safety in terms of antibiotic resistance and virulence factor involvement is the first step to introducing a microorganism as a food material, according to the European Food Safety Authority guidelines (EFSA, 2004). Therefore, in this study, we assessed the safety and technological properties of CNS isolates from meju and doenjang and selected a safe and functional starter candidate. Additionally, the volatile compounds produced by the growth of this starter candidate in sterilized soybeans were analyzed by headspace solid phase micro-extraction (SPME) coupled with gas chromatography–mass spectrometry (GC–MS) to predict its roles in soybean fermentation.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Forty-nine *S. saprophyticus* strains, 14 *S. succinus* strains, and 18 *S. xylosus* strains previously isolated from meju and doenjang (Jeong et al., 2014b) were used in the current study for safety assessment and technological characterization. The bacterial strains were mainly cultured in tryptic soy agar (TSA; Difco, Detroit, MI, USA) and tryptic soy broth (TSB; Difco) at 30 °C for 24 h to maintain their traits.

2.2. Antibiotic susceptibility test

For antimicrobial susceptibility testing, we used the disk diffusion method according to the guidelines for CNS set out by the Clinical and Laboratory Standards Institute (2007) and the European Committee on Antimicrobial Susceptibility Testing (2014). Test strains cultured in TSB were adjusted to the equivalent of a 0.5 McFarland turbidity standard (bioMerieux, Marcy l'Etoile, France), and then inoculated onto the entire surface of Mueller-Hinton agar plates (Oxoid, Basingstoke, Hants, UK) to form an even lawn of bacteria. Sterile paper disks were placed on the surface of each plate and incubated at 30 °C for 24 h. Eight types of antibiotic disks containing ampicillin (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (10 μg), lincomycin (15 μg), penicillin G (10 units), tetracycline (30 μg), and trimethoprim (5 μg) were purchased from Oxoid. All experiments were conducted three times on separate days using fresh strain cultures.

2.3. Hemolytic activity test

TSA supplemented with 5% rabbit blood (v/v) (MB cell, Daejeon, Korea) and 5% sheep blood (v/v) (BBL Microbiology Systems, Sparks, MD, USA) was used for α- and β-hemolytic activity tests, respectively. α-Hemolytic activity was determined by incubation at 30 °C for 24 h and β-hemolytic activity was determined by cold shock at 4 °C for 24 h after incubation at 30 °C for 24 h (Jeong et al., 2014a). Experiments were conducted at least three times, on separate days.

2.4. Biofilm formation assay

An overnight culture in TSB was diluted 200-fold with fresh TSB containing 0.5% glucose (w/v). A 200-μL aliquot of culture was then added to each well of a 96-well microtiter plate 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). Biofilm formation was determined by safranin staining. Experiments were conducted at least three times, on separate days.

2.5. Biogenic amine production analysis

Overnight cultures of each strain were each normalized to an optical density (OD) of 1.0 by measuring the OD at 600 nm, after which 1:100 dilutions of each normalized culture were inoculated into TSB containing 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); 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 2 days at 30 °C under semi-aerobic conditions, after which 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 any 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.

Biogenic amine production was determined using an Agilent Technologies HPLC 1200 series system (Palo Alto, CA, USA) monitored by UV detector at 254 nm, and a Nova-Pak C18 column (4 μm, 150 × 4.6 mm, Waters, Milford, MA, USA) for chromatographic separation. The gradient elution program began with 50:50 acetonitrile:0.1 M ammonium acetate (v/v) 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, which was decreased to 50:50 over the final 2 min. Four *Enterococcus faecalis* strains 7AME11, 7AME19, 0BML21 and 0AME15, were used as a positive control for tyramine production (Jeong et al., 2015). All experiments were conducted three times on independent samples prepared in the same way on separate days.

2.6. Determination of salt tolerance, acid production, and protease and lipase activities

The salt tolerance of CNS strains was determined by their growths on TSA supplemented with NaCl up to 21% (w/v). Growth on 12%, 18%, and 21% NaCl was determined after a 7-day incubation. Acid production was determined on TSA supplemented with 1% glucose (w/v) and 0.7% $CaCO_3$ (w/v). Protease activity was determined on TSA containing 2% skim milk (w/v), and lipase activity was tested on tributyrin agar (Sigma) containing 1% tributyrin (v/v). The tributyrin-supplemented medium was emulsified by sonication prior to autoclaving. Colonies cultured on TSA were transferred to each substrate-supplemented agar medium and incubated at 30 °C for 48 h. Activities were determined after detectable colonies appeared. The size of the zone of clearing around the colony was used as the indicator of enzyme activity. The effect of NaCl on each activity was determined by the addition of NaCl to each medium up to 15% (w/v) and the medium was incubated at 30 °C for 2 days up to 6% NaCl, and for 4 days for higher NaCl concentrations.

2.7. Inoculation of sterilized soybean with a starter candidate

Soybeans were washed, soaked overnight in water and autoclaved for 30 min at 121 °C. Logarithmic-phase cells cultured in TSB were inoculated into the prepared soybeans to approximately 5×10^5 colony forming units/g of sterilized soybeans, and then incubated at 25 °C for