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Probiotic characteristics of *Bacillus* strains isolated from Korean traditional soy sauce

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ABSTRACT

The objective of this work was to isolate *Bacillus* strains from Korean traditional soy sauces, and evaluate them for probiotic potential and safety. Three *Bacillus* strains, MKSK-E1, MKSK-J1 and MKSK-M1, were selected which were highly resistant to simulated gastrointestinal tract conditions, and showed antimicrobial activity against *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Escherichia coli*. MKSK-M1 possessed the highest relative survival rate, with 96.0% survival in 0.1% pepsin solution (pH 2.0) and 99.3% survival in 3% bile salt solution for 3 h. All three strains exhibited amylase and protease activity, and utilized more than 19 carbohydrates among 49 tested carbohydrates. MKSK-E1 inhibited the growth of 11 of 13 tested foodborne pathogens. All three selected strains possessed strong antimicrobial activity against *E. coli* and *B. cereus*. They had antibiotic susceptibility against 8 tested antibiotics. Also, all strains were non-hemolytic on sheep blood, and non-biogenic amine producers. The results of this work indicate that MKSK-E1, J1 and M1 could be used as probiotic cultures for human consumption and animal feeds and as probiotic starter cultures in food industries.

1. Introduction

The market for probiotics expanded by 5–30% in 2013, and is estimated to be about 12.5 billion dollar worldwide (Yang, 2014). Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit to the host" (Joint FAO/WHO, 2002). *Lactobacillus*, *Lactococcus*, *Bifidobacterium*, *Leuconostoc*, *Pediococcus*, *Bacillus* and many others are included in the list of probiotics (Isolauri, Salminen, & Ouwehand, 2004). Probiotics are well known to possess specific properties such as gastric juice and bile tolerance, adhesion to the epithelial cells of the intestine, and improvement of the intestinal microbial balance (Ministry of Food and Drug Safety, 2015a; Ouwehand, Salminen, & Isolauri, 2002). The main feature of probiotics is based on their antagonistic or antimicrobial activities against pathogenic bacteria in the intestine (Quigley, 2010). This is generally a result of bacteriocins secreted from probiotic cultures or competitive metabolic interactions between probiotics and pathogens (O'Hara & Shanahan, 2007; Zeng, Li, Zuo, Zhen, & Liu, 2008). Probiotics are also recognized to alleviate allergic symptoms as well as infectious and inflammatory diseases (Isolauri et al., 2004). The health functionality of probiotics provides opportunities for the development of health functional foods, animal feeds, medicine and cosmetic products (Hwang, Seo, & Cho, 2013; Um, 2010). In addition to health related products, probiotics are used with starter cultures or by themselves for the production of fermented foods such as dairy foods, *kimchi*, soybean fermented foods and fermented meats (Lee et al., 2010; Lee, 2011; Kim, 2013; Um, 2010).

These beneficial effects continuously cause researchers to seek new species and strains with more specific features as novel probiotic candidates (Ryu & Chang, 2013). However, like common microorganisms, probiotics may possess undesirable properties such as the advent of harmful biochemical and virulence factors

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and antibiotic resistance (Ammor, Flórez, & Mayo, 2007; Clementi & Aquilanti, 2011; Donohue, 2006). Therefore, the safety status of probiotics should be confirmed as well as their antibiotics resistance and hemolytic potential (Park & Ji, 2014).

Korean traditional soy sauce is a fermented condiment by soaking a naturally fermented soybean brick for several weeks in brine solution. The main microorganisms involved in the fermentation of Korean traditional soy sauce are *Bacillus* species such as *B. subtilis, B. licheniformis, B. pumilus*, and *B. amyloliquefaciens*. The members of genus *Bacillus* are spore forming bacteria, and their spores are able to survive extreme environmental conditions that are enough to kill vegetative bacterial cells such as high heat, low pH, dry and undernutrition environments (Barbosa, Serra, La Ragione, Woodward, & Henriques, 2005; Nicholson, Munakata, Horneck, Melsosh, & Setlow, 2000). If such spores are exposed to appropriate nutrients or conditions, they can be germinated for vegetative bacterial cells. Therefore, spore-forming probiotics, being highly stable in the low pH in the gastric barrier, have advantages over non-spore formers such as *Lactobacillus*, *Lactococcus*, *Bifidobacterium*, *Leuconostoc*, and *Pediococcus* (Cutting, 2011).

The objective of this work was to isolate probiotic strains from Korean traditional soy sauces and characterize their carbohydrate utilization, enzyme activity, antimicrobial spectrum, antibiotic sensitivities, hemolysis, and the formation of biogenic amines.

2. Materials and methods

2.1. Isolation and identification

Korean traditional soy sauces were purchased through internet markets from 17 regions. The method of screening for probiotic *Bacillus* strains was slightly modified from the method described by Patel, Ahire, Pawar, Chaudhari, and Chincholkar (2009). Briefly, 1 ml of soy sauce was heated at above 95 °C for 5 min to isolate *Bacillus* sp. It was incubated in 5 ml of 0.85% sterile saline solution containing 0.1% pepsin (pH 2.0) for 3 h at 37 °C and was adjusted to pH 6.0 using 0.1 M $NaHCO_3$. Sequential incubation was performed in 5 ml of 3% bile salt (Sigma Aldrich, St. Louis, USA) solution (12.4 g/l of K_2HPO_4, 10 g/l of trisodium citrate, 7.6 g/l of KH_2PO_4 and 6 g/l $(NH_4)_2SO_4$, pH 6.7) for 3 h at 37 °C. The complete reaction mixtures and their dilutions were spread onto nutrient agar (NA) plates containing 5 g/l of peptone, 3 g/l of beef extract and 15 g/l of agar, then incubated at 37 °C for 24 h. Colonies with different morphologies were randomly selected, and cultured in nutrient broth (NB) containing 5 g/l of peptone and 3 g/l of beef extract at 37 °C for 24 h for further screening.

These were also differentiated based on microscopic observation, the patterns of cell wall proteins by SDS-PAGE, carbohydrate utilization using API 50 CHB kits (BioMerieux, Lyon, France) and antimicrobial activity against *Bacillus cereus* KCTC 1012 (Korean Collection for Type Cultures, Daejeon, Korea), *Listeria monocytogenes* KCTC 3710, *Staphylococcus aureus* KCTC 3881 and *Escherichia coli* KCTC 1682.

All selected cultures were stored in equivalent mixed solutions of 40% (v/v) glycerol and NB until use. Selected isolates were identified using 16S rDNA sequence analysis by a commercial service (SolGent, Daejeon, Korea). The phylogenetic tree was generated by the neighbor-joining (NJ) method using MEGA 6 software (Tamura, Stecher, Peterson, Filipski and Kumar, 2013).

2.2. Enzymatic activity

The amylase activity of the selected isolates was determined using a slightly modified method from Gangadharan, Sivaramakrishnan, Nampoothiri, Sukumaran, and Pandey (2008). The supernatant (0.25 ml) of a 24 h culture obtained by centrifuging (Gyrozen, Seoul, Korea) at 9400×*g* for 3 min was reacted with 1.75 ml of 1% soluble starch solution (in 0.1 M acetate buffer, pH 5.0) at 50 °C for 30 min. Then, 0.2 ml of the reactant was mixed with 0.6 ml of DNS solution at 100 °C for 5 min, 3 ml of distilled water was added, and the mixture was stored in a darkroom for 15 min. Lastly, its absorbance was measured at 550 nm using a UV/visible spectrophotometer (GE healthcare, Buckinghamshire, UK). One unit of amylase activity was defined as the amount of enzyme that released 1 μg of glucose equivalent per min at 37 °C.

The protease activity of the selected *Bacillus* strains was assayed according to a slightly modified method from Lee et al. (2010). The supernatant (0.3 ml) of a 24 h culture was reacted with 0.3 ml of 1% casein solution (in 0.2 M sodium phosphate buffer, pH 3.7) at 37 °C for 10 min. The reaction was stopped by adding 0.6 ml of 0.4 M TCA solution, and centrifuged at 9400×*g* for 3 min. Supernatant (0.15 ml) was reacted with 0.75 ml of 0.4 M sodium carbonate and 0.15 ml of 3 × folin reagent (Sigma Aldrich, St. Louis, USA) at 37 °C for 20 min. The absorbance was measured at 600 nm using a UV/visible spectrophotometer. One unit of protease activity was defined as the amount of enzyme that released 1 μM of tyrosine equivalent per min at 37 °C.

The selected *Bacillus* strains were evaluated to determine their enzyme activity using a API ZYM kit (BioMerieux, Lyon, France).

2.3. Relative survival ratio

The relative survival ratio (RSR) of the selected *Bacillus* strains was measured with a slightly modified method from Argyri et al. (2013). Each 5 ml of a 24 h culture was incubated in 0.1% pepsin saline solution (pH 2.0) or 3% bile salt solution for 0, 1, 2 and 3 h at 37 °C in an air shaker (Hanbaek CO. LTD, Bucheon, Korea). After each reactant was spread onto NA plates and incubated for 24 h at 37 °C, viable cells against pepsin or bile salt solution were counted.

$$RSR = (\log CFU\ N_0 - \log CFU\ N_t)/\log CFU\ N_0 \times 100$$

N_o = Total viable contents for selected *Bacillus* strains before treatment.
N_t = Total viable contents for selected *Bacillus* strains after 1, 2 and 3 h in pepsin or bile salt.

2.4. Antimicrobial activity

The selected *Bacillus* strains were evaluated for antimicrobial activity against thirteen gram positive and negative bacteria. All pathogens were obtained from Korean Collection for Type Cultures (Daejeon, Korea). *Staphylococcus aureus* KCTC 3881, *Escherichia coli* KCTC 1682 and *Proteus vulgaris* KCTC 2512 were cultured on TSB (17 g/l of pancreatic digest of casein, 3 g/l of papaic digest of soybean, 2.5 g/l of dextrose, 5 g/l of sodium chloride, 2.5 g/l of dipotassium phosphate), and *Vibrio paraheamolyticus* KCTC 2729 was cultured on TSB containing 3% NaCl. *Bacillus cereus* KCTC 1012, *Listeria monocytogenes* KCTC 3710 and *Listeria innocua* KCTC 3586 were grown on BHI broth (5 g/l of beef heart infusion solid, 10 g/l of proteose peptone, 2 g/l of glucose, 5 g/l of sodium chloride and 2.5 g/l of di-sodium phosphate), *Staphylococcus epidermidis* KCTC 1917, *Pseudomonas aeruginosa* KCTC 1750, *Shigella sonnei* KCTC 2518, *Shigella flexneri* KCTC 22192, *Pseudomonas fluorescens* KCTC 1767 and *Klebsiella pneumoniae* KCTC 2208 were cultured on NB.

The antimicrobial activity was determined by a modified method from Argyri et al. (2013). Briefly, 5 μl of 24 h cultured