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Probiotic properties of *Weissella* strains isolated from human faeces

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ABSTRACT

Three *Weissella confusa* and five *Weissella cibaria* strains were previously isolated from human faeces and their potential as probiotics was examined in this work. Resistance to low pHs (pH 2.0 and 3.0) and 0.3% bile salt were examined. Enzyme activities, susceptibilities to heat treatment and various antibiotics, and adhesion capacities to Caco-2 cells were also examined. All *Weissella* strains were killed when exposed to pH 2.0 for 2 h but survived at pH 3.0 with different survival ratios. *W. confusa* 31 survived best (20.2%) and *W. confusa* 31 was also quite resistant against 0.3% bile salt (128.8%). All strains except one grew well at temperature between 15 and 45 °C and all strains grew in the presence of 6.5% NaCl. *W. confusa* 20 showed the highest β-galactosidase activity (527.3 ± 23.66 unit/mg protein) and *W. cibaria* 31 had the highest β-glucosidase activity (115.12 ± 5.3 unit/mg protein) in MRS broth. All strains adhered to Caco-2 cells better than *Lactobacillus rhamnosus* GG and *W. confusa* 20 was the best adhesive strain (85 CFU/cell). These results show that some strains such *as W. confusa* 31 and *W. confusa* 20 are fully qualified as probiotics and deserve further application studies.

1. Introduction

Probiotics are live microorganisms that are administered to human beings and farm animals for the purpose of improving the general health conditions of hosts [1,2]. Lactic acid bacteria (LAB) are the most important probiotics employed because of their long history of uses as starters for various fermented foods with few adverse effects on the health [3,4]. Importance of LAB has been increased recently because use of antibiotics for farm animals has been tightly controlled or banned in many countries [5,6]. Various antibiotics have been included into feed for farm animals to promote growth and prevent diseases. But antibiotic growth promoters (AGPs) have been suspected to be responsible for the appearance and spread of pathogens resistant against multiple antibiotics. Thus for the production of safe foods, effective alternatives such as probiotics must be used instead of AGPs. If an organism is to be used as a probiotic, the strain should possess some properties required for a successful probiotic in addition to no known pathogenicity. A probiotic strain should possess high level of resistance against low pHs and bile salts which ingested organisms inevitably encounter during the passage through stomach and intestines of human being [7]. Ability to efficiently adhere to epithelial cells in the intestines is also very important for organisms to establish successfully in the intestines [8]. Some enzyme activities of probiotic strains are believed to play some beneficial roles for the efficient utilization of ingested nutrients. For an example, β-glucosidases (β-Glu) of LAB have been used for the bioconversion of soy isoflavone glycosides into aglycones during soymilk fermentation [9]. The latter forms are known to be more readily absorbed in the intestines, thus more bioactive than their corresponding glycosides [10]. In another example, ginsenosides from *Panax ginseng* were transformed into deglycosylated forms by β-Glu from edible microorganisms [11]. Other enzymes such as α-amylases and α-galactosidases are also desirable for probiotics since these enzymes contribute to the improved utilization of nutrients in coarse feed by hosts [12].

Weissella species are relatively recent members of LAB. Its genus status was proposed in 1993 [13] and currently 14 species are officially recognized [14]. All *Weissella* species are nonspore formers, all Gram positive, catalase negative and they ferment glucose via heterolactic fermentation pathway, producing lactic acid and carbon dioxide [15]. *Weissella* species are distributed among various environments including fermented vegetables like *kimchi*, fermented foods, sugar cane, and gastrointestinal tracts of human and animals [15]. *Weissella* strains with β-Glu activities

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were previously isolated from human faeces and used as starters for fermented soymilk enriched in isoflavonoid aglycones [9]. In this report, we examined the probiotic properties of these *Weissella* strains for the purpose of evaluating their potential as probiotics. We found that some of them were qualified as probiotics.

2. Materials and methods

2.1. Isolation and identification of Weissella strains

LAB with β-Glu activities were previously isolated from human faeces [9]. Faecal samples from college students (Gyeongsang Nat'l. Univ., Jinju, Korea) were serially diluted with 0.1% peptone water. Diluted samples were spread on deMan Rogosa Sharpe (MRS, Difco, Becton Dickinson Co., Sparks, MD) agar plates with cellobiose (1%, w/v) as the carbon source and incubated at 30 °C using an anaerobic Jar, BBL Gaspak system™ (BD, Franklin lakes, NJ). Big colonies were selected and streaked on MRS agar plates to isolate each colony. Identification of selected colonies was done first by using API CHL50 kit (BioMerieux, Marcy L'Etoile, France) and then by 16S rRNA gene sequencing. The universal primer set was used to amplify 700 base pair (bp) 16S rRNA gene fragment: Leu1 (5′-GCGGCGTGCCTAATACATGCAAGTCG-3′) and Leu2 (5′-GACCCGGGAACGTGTTCACCGCGGC-3′) [16]. Sequence alignment search tool (BLAST) provided by National Center for Biotechnology Information (NCBI, Bethesda, MD) was used for homology analyses. *recA* gene sequencing was done to confirm the identification by 16S rRNA gene sequencing. Primer pairs to amplify ca 1100 bp *recA* gene fragment were as follows: recAF (5′-GGAAGGAAAGATTGCAGACC-3′) and recAR (5′-GTTATCAGCTGAC GTGTCG-3′). The primer sequences were based on the *recA* gene sequence of *Weissella cibaria* KACC11862 for which the genome sequencing was recently completed (personal communications).

2.2. Growth of Weissella strains

Weissella strains were grown in MRS broth at 30 °C without shaking. Growth on different carbon source (glucose, lactose, galactose, and sucrose, 1%, w/v) was examined by measuring the optical density at 600 nm (OD_{600}) of each culture. Each *Weissella* strain was first propagated in 10 ml MRS broth (glucose 2%) for 24 h at 30 °C and then inoculated into fresh MRS broth (1%, v/v) with different carbon sources. Each *Weissella* strain was cultivated in MRS broth at 4 °C, 15 °C, 37 °C, and 45 °C for 48 h and the growth was examined by measuring OD_{600} values at time points.

2.3. Enzyme assays of Weissella strains

β-Glu activity was determined by the method of Belancic et al. [17] and para–nitrophenyl–β–D–glucopyranoside (pNPG) was used as the substrate. After cells were disrupted by sonication, β-Glu activity of cell extract was determined by incubating mixture consisting of 50 μl of pNPG (10 mM), 50 μl of sodium acetate (200 mM, pH 5.5) and 0.1 ml of cell extract at 37 °C for 15 min. The reaction was stopped by adding 800 μl of 1 M sodium carbonate. The amount of p-nitrophenol released was measured at 400 nm using a spectrophotometer. One unit of β-Glu activity was defined as the amount of enzyme that released 1 μmol of p-nitrophenol from the substrate per minute.

β-galactosidase (β-Gal) activities of *Weissella* strains were measured using the method of Miller [18]. *Weissella* strains were grown in 10 ml of MRS broth until the OD_{600} reached 1.0. Cells were collected by centrifugation and resuspended in 1 ml of Z buffer (60 mM Na_2HPO_4–$7H_2O$, 40 mM NaH_2PO_4–H_2O, 10 mM KCl, 1 mM $MgSO_4$–$7H_2O$, 50 mM β-mercaptoethanol, pH 7.0), placed on ice and disrupted by sonication (30 s, 6 times) using a homogenizer (Bandelin, Berlin, Germany). 500 μl of disrupted cells were incubated with 200 μl of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) solution (4 mg/ml in A buffer; K_2HPO_4 1.05 g, KH_2PO_4 0.45 g, $(NH_4)_2SO_4$ 0.1 g, Na_3-citrate-$2H_2O$ 0.05 g, 100 ml final volume) at 28 °C until yellow color appeared. Reaction was stopped by the addition of 500 μl of 1 M sodium carbonate. After centrifugation at 12,000 rpm for 10 min, the absorbance of supernatant was measured at 420 nm and 550 nm. β-Gal units were calculated according to the following equation:

$$\beta - \text{Gal unit} = [A_{420} - (1.75 \times A_{550})]/t \times A_{600}$$

t: time in min taken for yellow color development.

α-galactosidase and α-amylase activities were measured by the method of Church et al. [19] and Bernfeld [20], respectively. First, *Weissella* strains were grown in MRS broth until OD_{600} reached 1.0. Then cells were disrupted by sonication. p-nitrophenyl-α-galactopyranoside (pNPGal) was used as the substrate for α-galactosidase and the released p-nitrophenol (pNP) was measured at 400 nm. Reaction mixture for α-amylase assay consisted of 0.5 ml of cell extract, 2 ml of acetate buffer (pH 5.4), 5 ml of soluble starch (Sigma), 1 ml of 1% NaCl, and 0.5 ml of distilled water, and incubated at 65 °C for 30 min. The amount of released reducing sugar was measured by dinitrosalicylic acid (DNS) method [21].

2.4. Stress resistance of Weissella strains

Weissella strains were cultivated in MRS broth until the OD_{600} reached 1.5. One ml of culture was centrifuged at 12,000× *g* for 5 min at 4 °C and cell pellet was resuspended in MRS broth where the pH of MRS broth was previously adjusted to 2, 3, or 6.5 (control), respectively, by 1 N hydrochloric acid. Cell suspensions in different pHs were incubated for 2 h at 30 °C. Then viable cells were counted by standard plate counting. Measurements were done in triplicates and the mean values were shown.

Tolerance of *Weissella* strains against bile salts was determined using MRS broth with 0.3% bile salts (cholic acid sodium salt 50% and deoxycholic acid sodium salt 50%, Fluka, cat no 48305). *Weissella* cells grown overnight in MRS broth were harvested by centrifugation, washed and resuspended with 1 ml of MRS broth supplemented with 0.3% bile salts. After 2 h incubation at 30 °C, viable cells were counted.

Heat resistance of *Weissella* strains was tested by the method of Stopforth et al. [22]. *Weissella* strains were cultivated in MRS broth until OD_{600} reached 1.5. One ml of the culture was centrifuged for 5 min at 4 °C and cell pellet was resuspended in phosphate buffered saline (PBS, pH 7.4). Cells were exposed to different temperature (60, 70 or 80 °C) for 1, 3, or 5 min. After heat treatment, cells were immediately cooled on ice and the viable cells were counted.

Tolerance against NaCl was examined using MRS broth with 6.5% and 8% NaCl. *Weissella* culture previously grown in MRS for 24 h was used to inoculate fresh MRS broth with salt (1% inoculum, v/v). Inoculated broth was incubated for 48 h at 30 °C and growth of each *Weissella* strain was monitored by measuring OD_{600} values.

2.5. Antibiotic susceptibility assays

Antibiotic susceptibility of *Weissella* strains was examined by agar overlay diffusion method as described by Cebeci and Gurakan [23]. MRS agar plates were overlaid with 4 ml of soft agar (0.7%, w/v) containing 200 μl of indicator culture (10^8 CFU/ml) and then stood for 1 h at room temperature. Then paper discs were overlaid on the plates and antibiotics were applied onto the discs. Plates were incubated under anaerobic conditions for 24 h at 30 °C. The