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Screening probiotic strains for safety: Evaluation of virulence and antimicrobial susceptibility of enterococci from healthy Chinese infants

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ABSTRACT

The aim of this study was to evaluate the safety of enterococci isolated from Chinese infants and screen out potential probiotic candidates. One hundred eight strains were isolated from feces of 34 healthy infants, and 38 strains of *Enterococcus* spp. were categorized as follows: *E. faecalis* (22), *E. faecium* (10), *E. hirae* (3), *E. durans* (2), and *E. casseliflavus* (1). Of these, 72.7% of *E. faecalis* came from infants delivered by cesarean and 62.5% of *E. faecium* from infants delivered vaginally. For safety evaluation of strains, we determined presence of virulence genes; production of hemolysin, gelatinase, and biofilm; and antimicrobial susceptibility of enterococci. Six out of 14 virulence genes were detected with a distribution of *gel*E (26.3%), *cyl*A (39.4%), *esp* (15.8%), *efa*A (63.2%), *asa*1 (50.0%), and *ace* (50.0%). In phenotype analysis, 36.8% of the strains exhibited positive hemolytic activity and 17.5% were positive for production of gelatinase. Results of antimicrobial susceptibility showed that different percentages of the strains were resistant to ciprofloxacin (5.2%), vancomycin (7.8%), rifampicin (10.5%), erythromycin (52.6%), and gentamycin (52.6%); remarkably, none of the strains were resistant to ampicillin or chloramphenicol. In total, 10 strains, including 6 *E. faecium*, which are free of virulence determinants and sensitive to common antimicrobial agents (e.g., ampicillin and vancomycin), were further assessed for their probiotic properties. All strains survived well in simulated gastric fluid and intestinal tract, with maximum reductions of 0.600 and 0.887 log cfu/mL, respectively. Six strains of *E. faecium* could resist 0.3 to 1.0% bile salt, of which *E. faecium* WEFA23 presented the highest growth (75.06%) at 1.0% bile salt. All strains showed bile salt hydrolase activity on glycodeoxycholic acid, but only 3 of *E. faecium* showed activity on taurodeoxycholic acid. These results deliver useful information on the safety of enterococci in infants in China, and provide a protocol to screen probiotics for absence of virulence and antimicrobial susceptibility of enterococci.

Key words: enterococci, safety, probiotic, virulence, antibiotic susceptibility

INTRODUCTION

Enterococci are gram-positive, catalase-negative cocci that are ubiquitous in a variety of food and dairy products, water surfaces and plants, and are natural inhabitants in gastrointestinal tract (**GIT**) of humans and animals (Gelsomino et al., 2002; Ahmed et al., 2012). Belonging to the lactic acid bacteria (**LAB**), enterococci have shown beneficial effects; for example, restoration of microbiota balance of GIT with antibiotic-induced dysbiosis (Tarasova et al., 2010), antiviral activity (Wang et al., 2013), antitumor protective effect (Castro et al., 2010; Thirabunyanon and Hongwittayakorn, 2013), ability to lower serum cholesterol level (Guo et al., 2015), and immune regulation (Molina et al., 2015).

However, the application of enterococci as probiotics remains controversial due to the association of these bacteria with nosocomial infections and multiple antibiotic resistance. Concerns about the dual role of enterococci as beneficial organisms and opportunistic pathogens have led to an increasing number of studies intended to discriminate food-grade and pathogenic strains (Pérez-Martín et al., 2014; Araújo et al., 2015; Corcuera et al., 2015). Enterococci are among the first LAB to colonize the neonatal GIT (Fanaro et al., 2003) and could be associated with infant health and development of the human microbiome (Dominguez-Bello et al., 2010). Therefore, verifying the safety of enterococci from infants is of utmost importance. Kirtzalidou et al. (2012) focused on enterococci from Greek healthy in-

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fants to enlighten the spread of resistant enterococci in the infant gut microbiota. Hufnagel et al. (2007) found that colonization of newborn infants with enterococci and drug-resistant enterococci increased in preterm infants. Werner et al. (2012) reported that vancomycin-resistant *Enterococcus faecium* isolates were highly prevalent among neonatal patients.

To the best of our knowledge, no study has been carried out in China to characterize enterococci in terms of their safety, and the beneficial properties of enterococci isolated from infants have seldom been reported. In this study, 38 enterococci isolates obtained from feces of 34 healthy Chinese infants were genetically characterized and assayed for their virulence potential and antimicrobial susceptibility. The isolates that did not harbor antibiotic resistance or virulence factors were further assessed for survival under simulated gastrointestinal tract and by bile salt challenge, and bile salt hydrolase (**BSH**) activities were assessed.

MATERIALS AND METHODS

Feces Samples of Infants

Fecal specimens were collected from 34 healthy infants from Maternal and Child Health Hospital of Jiangxi province (China). All infants were 1 to 6 d old (19 male and 15 female); 21 were born by cesarean delivery (C-section) and 13 by vaginal delivery. All were bottle and breast fed and none had a history of antimicrobial treatment. Mothers were made aware of the nature of the study and provided their personal information. The study was reviewed and approved by the Ethics Committee of the Jiangxi Maternal and Child Health Hospital (EC Number: 2014008).

Isolation and Identification of Enterococcus

Fecal specimens were collected in sterile centrifuge tubes and were homogenized in PBS, at pH 7.0. Then the specimens were properly diluted and plated on de Man, Rogosa, and Sharpe agar (Beijing Solarbio Science and Technology Co. Ltd., Beijing, China) plates. These plates were incubated under anaerobic conditions (5% CO_2, 10% H_2, and 85% N_2) for 48 h at 37°C. The isolates were screened by Gram staining and catalase activity, and the gram-positive and catalase-negative isolates were further screened by PCR using the primers Ent1 and Ent2 (Todorov et al., 2010). The selected isolates were then subjected to 16S rDNA sequencing with universal primers 27F and 1492R (Tan et al., 2013). The amplicons were sequenced by Sangon Biotech Ltd. (Shanghai, China), and compared with the National Center for Biotechnology Information database by using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine their classification.

Safety Evaluation

Genetic Screening for Virulence Genes. Genomic DNA of the enterococci was extracted using the bead-beating method (Li et al., 2014). All strains were investigated by PCR for the presence of genes involved in the expression of insertion element (IS*16*), gelatinase (*gel*E), cytolysin (*cyl*A), hyaluronidase (*hyl*), aggregation substance (*asa*1), enterococcal surface protein (*esp*), endocarditis antigen (*efa*A), collagen adhesion (*ace*), vancomycin resistance–related genes (*van*A and *van*B), and genes for amino acid decarboxylases: histidine decarboxylase (*hdc*1 and *hdc*2), tyrosine decarboxylase (*tdc*), and ornithine decarboxylase (*odc*); the primers used are listed in Table 1.

Gelatinase and Hemolysis Activity. Gelatinase activity was determined by spotting 2-μL aliquots of the 18-h cultures on Luria Bertani agar (Oxoid Ltd., Basingstoke, UK) supplemented with 3% gelatin (wt/vol). After incubation for 24 h at 37°C, the presence of opaque halos surrounding the colonies indicated gelatinase production. Hemolysis activity was examined by streaking the 18-h cultures on brain-heart infusion (**BHI**) broth (Oxoid Ltd.) supplemented with 5% (vol/vol) defibrinated sheep blood (Ruite Co. Ltd., Guangzhou, China). After incubation for 24 h at 37°C, hemolysis activity was classified as α-hemolysis (greenish halo surrounding colonies), β-hemolysis (clear halo surrounding colonies), and γ-hemolysis (absence of clearing zone surrounding colonies). α-Hemolysis or β-hemolysis indicated positive hemolytic activity, and γ-hemolysis was taken as a negative result.

Biofilm Formation. The ability to form biofilms was evaluated by the method described by Kopit et al. (2014). Briefly, strains were incubated in BHI broth at 37°C for 24 h. Then, the cell suspension (20 μL) and fresh BHI broth (180 μL) were added to sterile 96-well polystyrene microtiter plates. A negative control was performed with 200 μL of BHI alone. After growth of the strains at 37°C for 24 h, triplicate wells were washed with 200 μL of PBS, inverted for 15 min to dry, and stained with 200 μL of 1% crystal violet for 15 min. Then, the wells were washed twice with PBS and filled with 200 μL of acetone and ethanol solution (20:80, vol/vol). The optical density (**OD**) at 595 nm was determined by using a microplate reader (Molecular Devices Co., Sunnyvale, CA). The cut-off OD (**ODc**) was defined as 3 standard deviations above the mean OD of the negative control. The ability of to form a biofilm was classified by Pieniz et al. (2015) as follows: OD $\leq$ ODc as non-biofilm-producer (0), ODc