Probiotic properties and adsorption of *Enterococcus faecalis* PSCT3–7 to vermiculite

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The probiotic properties of *Enterococcus* (*E.*) *faecalis* PSCT3–7, a new strain isolated from the intestines of pigs fed dietary fiber containing 50% sawdust, were investigated. *E. faecalis* PSCT3–7 tolerated a pH range of 3 to 8 and 0.3% bile salts, and it inhibited the growth of *Salmonella* Typhimurium in a concentration-dependent manner. In addition, *E. faecalis* showed resistance to several antibacterial agents. Vermiculite, a nutrient and microbial carrier, increased the bile tolerance of the strain. Scanning electron microscope images revealed good adsorption of *E. faecalis* PSCT3–7 onto vermiculite. *E. faecalis* PSCT3–7 represents a potential probiotic candidate to administer with vermiculite to swine.

**Keywords:** *Enterococcus faecalis*, *Salmonella* Typhimurium, lactic acid bacteria, probiotics, swine

The microbial community in the gastrointestinal tract of pigs adapts to high levels of dietary fiber. High-fiber diets increase, through time, the number of cellulolytic bacteria in the intestines of pigs [1,12,15], and pigs fed high-fiber diets will adapt to digest non-starch polysaccharides within 3 to 5 weeks [11].

In ongoing feed-trial studies, we screened several lactic acid bacteria from pigs fed different sources and levels of dietary fiber and undertook morphological, biochemical, and molecular characterization of strains with potential probiotic properties [9,10]. The first aim of the current study was to determine the probiotic properties of a new strain characterized as *Enterococcus* (*E.*) *faecalis* and designated as *E. faecalis* PSCT3–7. Probiotics administered to animals are usually incorporated to carrier medium. Vermiculite, an important support and carrier medium for a range of nutrients for animals, is also reported to create a protective envelope around microorganisms [5,14]. Therefore, our second aim was to examine the properties of *E. faecalis* PSCT3–7 adsorbed onto vermiculite.

The strain investigated in this study was taken from 23 lactobacilli strains we recently screened from the intestines of pigs fed dietary fiber sources containing 50% sawdust for 4 weeks (Unpublished). The strain was selected as it exhibited the greatest activity of digestive enzymes, including protease, cellulase, phytase, and α-amylase, based on API ZYM system results. Identification of *E. faecalis* PSCT3–7 was based on morphological and biochemical characterization using a negative catalase reaction, a positive PY R test (Murex Diagnostika, Germany), and the API 20 Strep system (bioMérieux, Germany) as described previously [16]. Its identity was confirmed by MALDI-TOF mass spectrometry and 16S rRNA sequence analysis and comparison with sequences available in the GenBank database using the BLAST algorithm (National Center for Biotechnology Information, USA). Sequence comparisons done by the Korean Culture Center of Microorganisms (Korea) showed 100% similarity of the strain to *E. faecalis* with the best sequence match with *E. faecalis* EU708623 (Fig. 1).

Fermentation experiments were carried out in lactobacilli MRS medium as described previously [9]. In a 48 h study, pH
Fig. 1. Phylogenetic tree showing sequence-based identification of *Enterococcus faecalis* PSCT3-7.

Fig. 2. Growth characteristics of *Enterococcus* (*E.*) *faecalis* PSCT3-7. Changes in pH (A), dissolved oxygen (DO) (B), optical density at 600 nm (OD$_{600}$) (C), and colony-forming unit per milliliter (CFU/mL) (D) during 48 h fermentation of *E. faecalis* PSCT3-7. Growth of *E. faecalis* PSCT3-7 (E) and vermiculite-adsorbed *E. faecalis* PSCT3-7 (F) in pH-adjusted broth for 2 h at 37°C. The experiments were performed in triplicate (n = 3).

and dissolved oxygen (DO) were automatically monitored, and the growth of cells was monitored by measuring the optical density at 600 nm (OD$_{600}$). Bacterial counts were performed by using one milliliter samples taken throughout the incubation period. During the 48 h incubation period, the pH of the culture gradually decreased (panel A in Fig. 2) and the level of DO sharply fell from 17 mg/L to 5.3 mg/L during the first 12 h, increased thereafter, and reached 18 mg/mL by 48 h (panel B in Fig. 2). The OD$_{600}$ results showed a sharp increase, peaked at 12 h, and remained stable and high until 48 h (panel C in Fig. 2).
Similar to the OD$_{600}$ changes, *E. faecalis* PSCT3-7 showed an initial exponential growth followed by a stationary phase (panel D in Fig. 2).

Acid and bile tolerance tests were performed as described previously [9], in pH-adjusted medium (pH 2–8) or by adding 0.3% bile salts (Sigma, USA) in the medium. *E. faecalis* PSCT3-7 tolerated a pH of 3 and grew well in a pH range of 3 to 8 for 2 h (panel E in Fig. 2). Intestinal contents or feed matrix may prevent exposure of probiotic bacteria to bile thereby increasing the survival and functioning of the organism in the gastrointestinal tract [3]. Hence, we tested whether adsorption to vermiculite may improve the acid and bile tolerance of *E. faecalis* PSCT3-7 by adding 1% (w/v) vermiculite in the media and found no enhancement in acid tolerance of the strain (panel F in Fig. 2). However, while *E. faecalis* PSCT3-7 showed viability for 3 h in medium containing 0.3% bile salts, a longer survival (up to 6 h) of the strain was observed when 1% vermiculite was added in the bile-containing medium (data not shown). The survival and growth of *E. faecalis* PSCT3-7 over a wide range of pH and in media containing bile salts suggest that the strain can survive and grow both in the acidic environment of the stomach and in the presence of intestinal bile salts.

The inhibitory activity of *E. faecalis* PSCT3-7 (starting inoculum: $10^5$, $10^7$ or $10^9$ colony-forming unit [CFU]/mL) against *Salmonella enterica* serotype Typhimurium (*Salmonella Typhimurium*, KCTC 2515, starting inoculum: $10^3$ or $10^6$ CFU/mL to model early and late infections in pigs) was determined by performing co-culture experiments in triplicate. A one-way ANOVA with Dunnett’s *post hoc* analysis was performed for *Salmonella* Typhimurium CFU/mL obtained after incubation.

**Fig. 3.** Inhibitory activity of *Enterococcus* (*E.*) *faecalis* PSCT3-7 against *Salmonella* Typhimurium growth. Co-culture of *E. faecalis* PSCT3-7 with *Salmonella Typhimurium* at initial inoculum of $10^5$ CFU/mL (A) and $10^6$ CFU/mL (B) showing *E. faecalis* PSCT3-7 concentration-dependent inhibition of *Salmonella Typhimurium* growth. (C and D) The minimum level of *E. faecalis* PSCT3-7 required to inhibit growth of *Salmonella Typhimurium* by 50% ($IC_{50}$) when *Salmonella Typhimurium* was inoculated at $10^5$ CFU/mL (C) and $10^6$ CFU/mL (D) initial levels. The experiments were performed in triplicate (n = 3). *p < 0.05 compared to the results obtained with control.
without or with 3 levels of *E. faecalis* PSCT3-7. Significantly lower *Salmonella Typhimurium* CFU/mL (*p* < 0.05) was observed between the highest *E. faecalis* PSCT3-7 levels (10^9 CFU/mL) and all other treatments, both at 6 and 12 h post-incubation. While *Salmonella Typhimurium* CFU/mL continuously decreased in co-cultures with 10^5 CFU/mL *E. faecalis* PSCT3-7, *Salmonella Typhimurium* cell counts reached more than 10^10 CFU/mL by 12 h in experiments with out or 10^5 or 10^7 CFU/mL *E. faecalis* PSCT3-7 (panels A and B in Fig. 3). The CFU/mL of *Salmonella Typhimurium* in the absence or presence of different levels of *E. faecalis* PSCT3-7 was fitted with the inhibitor-versus response model built in GraphPad Prism (GraphPad Software, USA), with the equation: 

\[ Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + \left(\frac{X}{\text{IC}_{50}}\right)} \]

where Top and Bottom are *Salmonella Typhimurium* CFU/mL in the absence of *E. faecalis* PSCT3-7 and at maximum growth inhibition in the presence of *E. faecalis* PSCT3-7, and IC_{50} is the minimum level of *E. faecalis* PSCT3-7 required to inhibit the growth of *Salmonella Typhimurium* by 50% in the co-culture experiments. The IC_{50} values were determined to be 1.39 × 10^8 and 2.39 × 10^8 CFU/mL for *Salmonella Typhimurium* inocula of 10^5 and 10^6 CFU/mL, respectively (panels C and D in Fig. 3). Mechanisms for inhibition of *Salmonella Typhimurium* growth by *E. faecalis* PSCT3-7 remain to be described. However, it has been reported that *E. faecalis* strains adhere to intestinal cells and produce antimicrobial substances [7,13], suggesting that *E. faecalis* PSCT3-7 may remain the dominant microflora in intestines; thereby, preventing invasion by *Salmonella Typhimurium* strains of infected animals. Furthermore, addition of vermiculite in the culture medium did not affect the antibacterial activity of *E. faecalis* PSCT3-7 against *Salmonella Typhimurium* (data not shown), suggesting that vermiculite could be used as a potential carrier for administration of *E. faecalis* PSCT3-7 to pigs.

Minimum inhibitory concentrations (MIC) of several antibacterial agents against *E. faecalis* PSCT3-7 were determined according to Clinical and Laboratory Standards Institute guidelines and by using *Staphylococcus aureus* and *Escherichia coli* quality control strains [4]. *E. faecalis* PSCT3-7 showed resistance to several antibacterial agents with MIC (μg/mL) values of greater than 512 (colistin, spectinomycin, streptomycin), 256 (chloramphenicol, florfenicol, norfloxacin, novobiocin), 128 (cephalexin), 64 (bacitracin, marbofloxacin), and 32 (gentamycin), while it was susceptible to amoxicillin at a MIC value of 1 μg/mL. This resistance pattern may be considered advantageous because probiotic strains with antibiotic resistance could be useful for restoring gut microbiota when administered to animals that are undergoing antibiotic treatment [8].

The ultrastructural morphology of *E. faecalis* PSCT3-7 was studied by using a scanning electronic microscope (SEM; models S-4300 and EDX-350; Hitachi, Japan). Sample preparations were essentially as described previously [6,16]. The SEM images revealed that *E. faecalis* PSCT3-7 is spherical or oval and is divided with perfect symmetry (panel A in Fig. 4). In addition, binary fission of the bacteria was evident in the SEM images.

Probiotics prepared as feed additives should have stability and longevity to ensure extended physiological activity. This can be achieved by encapsulation or by using carrier medium, such as vermiculite [2]. Adsorption of *E. faecalis* PSCT3-7 onto vermiculite was studied by using a SEM. Vermiculite-adsorbed *E. faecalis* PSCT3-7 was prepared according to a previous study.
method [16]. Panel B in Fig. 4 shows the typical silicate clay with porous and lamellar structures of expanded vermiculite. SEM images show good adsorption capacity of E. faecalis PSCT3-7 onto 1% vermiculite with several cells adsorbed on the surface of the plate and inside the porous structures (panel C in Fig. 4).

In summary, E. faecalis PSCT3-7 possesses the essential characteristics of a potential probiotic bacterial strain, including the ability to survive in bile salts and at a low pH. Moreover, it has antibacterial activity against Salmonella Typhimurium and good adsorption onto vermiculite. Future studies will further establish the clinical functionality of the strain in piglets challenged with enteric bacteria.

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Conflict of Interest

The authors declare no conflicts of interest.

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