Short Communication

J Vet Sci 2017, 18(1), 95-99 · https://doi.org/10.4142/jvs.2017.18.1.95

JVS

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

Jin-Yoon Kim[1], Elias Gebru Awji[2,†], Na-Hye Park[1], Ji-Yong Park[1], Jong-Choon Kim[3], Sam-Pin Lee[4], Joo-Won Suh[5,*], Seung-Chun Park[1,*]

[1]*Laboratory of Veterinary Pharmacokinetics and Pharmacodynamics, College of Veterinary Medicine, Kyungpook National University, Daegu 41566, Korea*
[2]*Lovelace Respiratory Research Institute, Albuquerque, NM 87108, USA*
[3]*Laboratory of Veterinary Toxicology, College of Veterinary Medicine, Chonnam National University, Gwangju 61186, Korea*
[4]*Department of Food Science and Technology, Keimyung University, Daegu 42601, Korea*
[5]*Center for Nutraceutical and Pharmaceutical Materials, Department of Bioscience and Bioinformatics, Myongji University, Yongin 17058, Korea*

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 PYR 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 Biothechnology 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

Received 18 Jan. 2016, Revised 9 May 2016, Accepted 8 Jun. 2016
*Corresponding authors: Tel: +82-53-950-5964; Fax: +82-53-950-5955; E-mails: parksch@knu.ac.kr (SC Park), jwsuh@mju.ac.kr (JW Suh)
†Present address: Department of Pharmacotherapy and Experimental Therapeutics, UNC Eshelman School of Pharmacy, Chapel Hill, NC 27599, USA
pISSN 1229-845X
eISSN 1976-555X

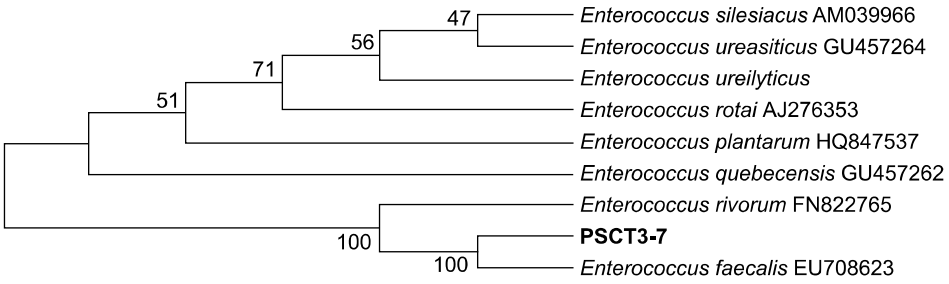

Fig. 1. Phylogenetic tree showing sequence-based identification of *Enterococcus faecalis* PSCT3-7.

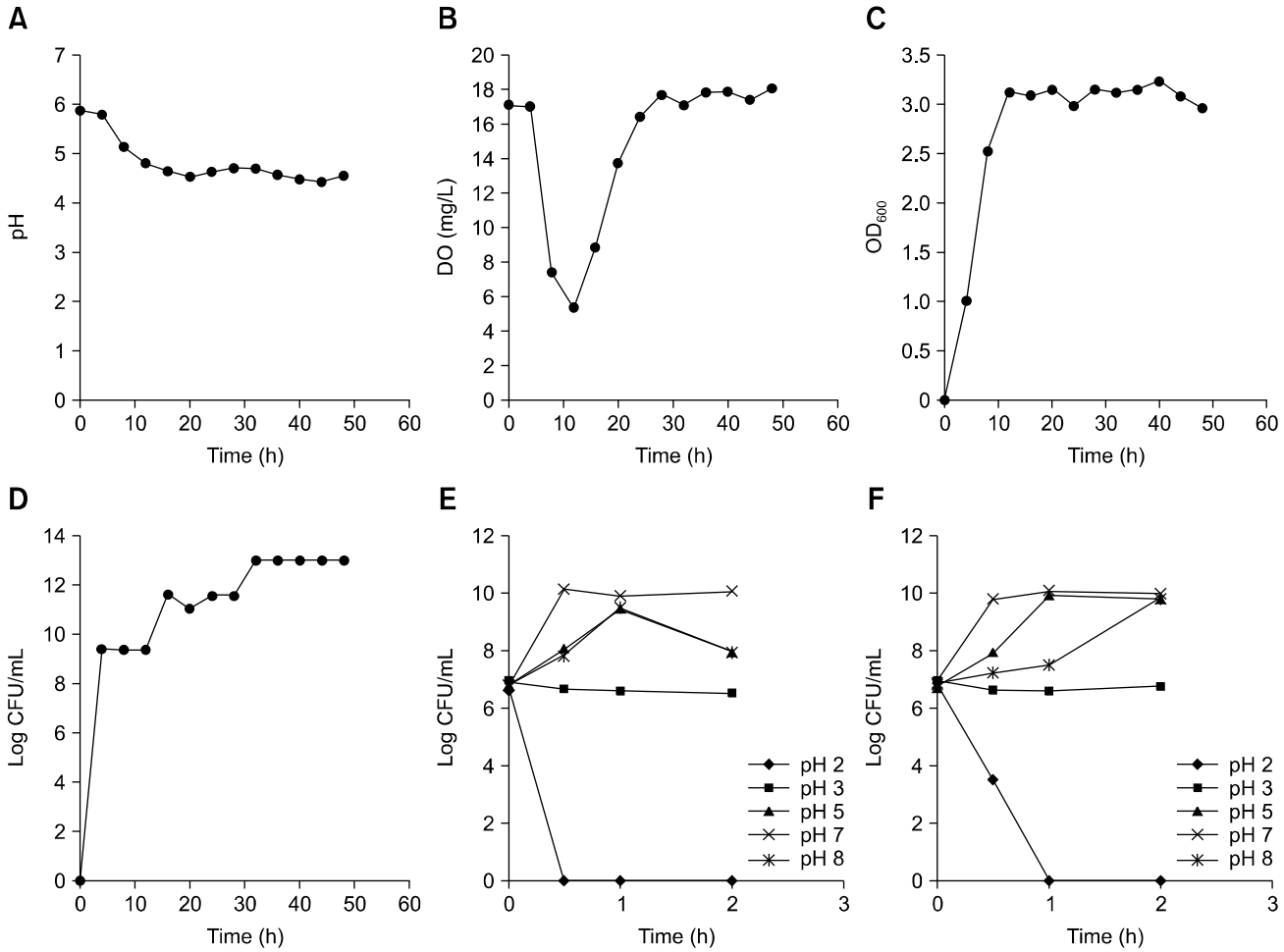

Fig. 2. Growth characteristics of *Enterococcus* (*E.*) *faecalis* PSCT3-7. Changes in pH (A), dissolved oxygen (DO) (B), optical density at 600 nm (OD_{600}) (C), and colony-forming unit per milliliter (CFU/mL) (D) during 48 h fermentation of *E. faecalis* PSCT3-7. Growth of *E. faecalis* PSCT3-7 (E) 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

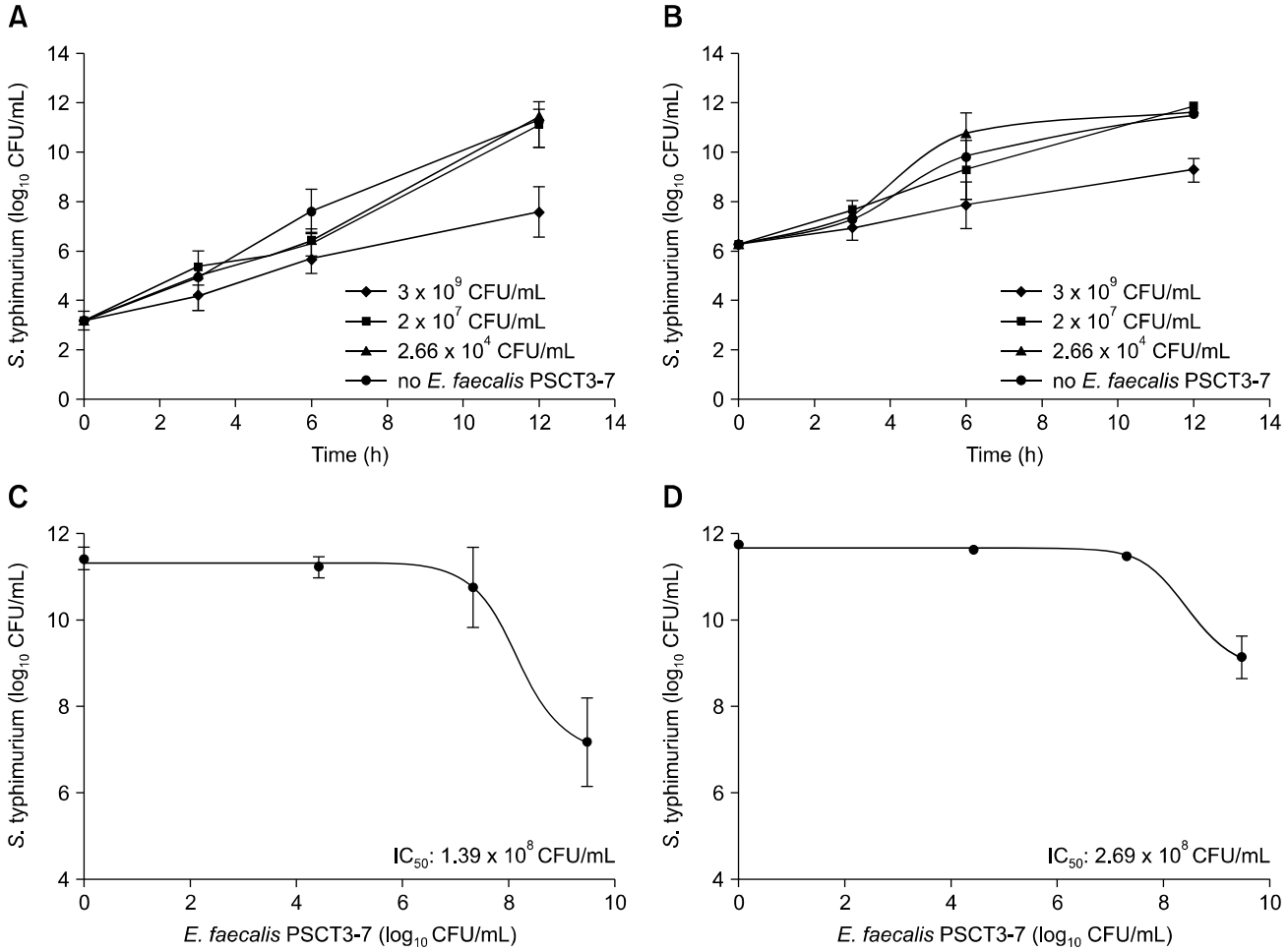

Fig. 3. Inhibitory activity of *Enterococcus* (*E.*) *faecalis* PSCT3-7 against *Salmonella* Typhimurium growth. Co-culture of *E. faecalis* PSCT3-7 with *Salmonella* Typhimurium at initial inoculum of 10^3 CFU/mL (A) and 10^6 CFU/mL (B) showing *E. faecalis* PSCT3-7 concentration-dependent inhibition 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 10^3 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 10^9 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 10^4 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: $Y = Bottom + (Top - Bottom)/(1 + (X/IC_{50}))$, 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 10^3 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

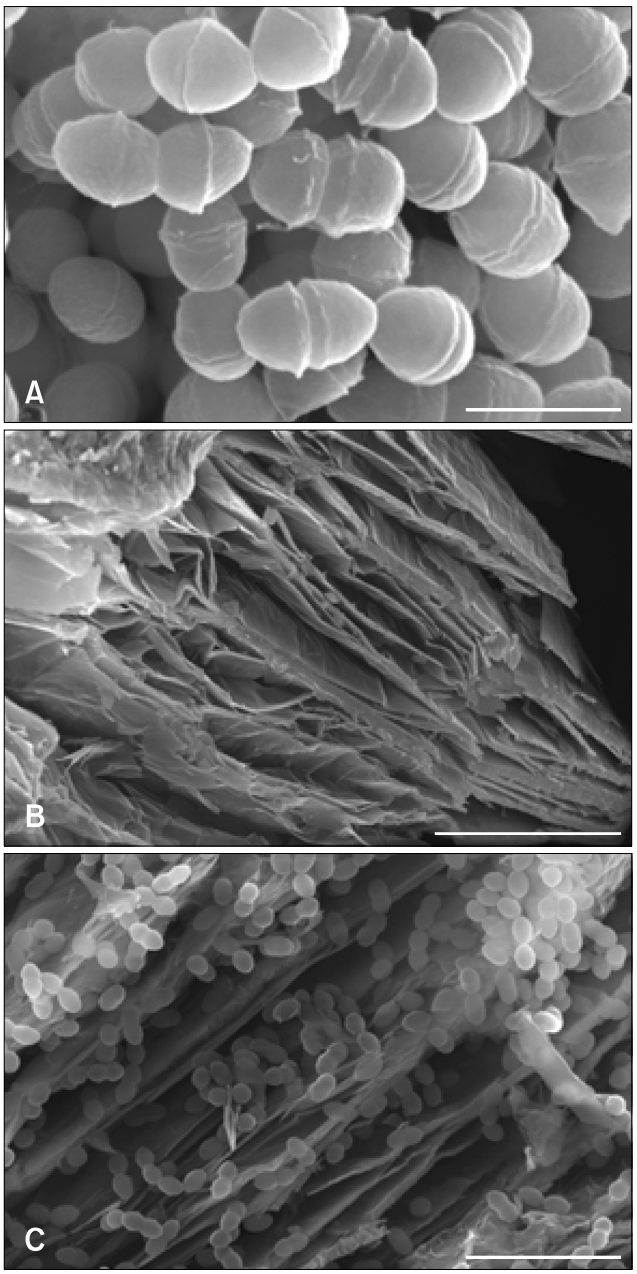

Fig. 4. Scanning electron microscope images of (A) *Enterococcus* (*E.*) *faecalis* PSCT3-7, (B) vermiculite, and (C) vermiculite-adsorbed *E. faecalis* PSCT3-7. Scale bar = 1 μm (A), 30 μm (B), 5 μm (C).

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.

Acknowledgments

This work was supported in part by the Korean Institute of Planning and Evaluation for Technology (IPET) through the Technology Commercialization Support Program (314082-3), funded by the Ministry of Agriculture, Food and Rural Affairs, in part by the Daejeon IRPE Project (R0004266) through the Research and Development for Regional Industry of the Ministry of Trade, Industry and Energy, and in part by the Cooperative Research Program for Agriculture Science & Technology Development (project No. PJ01128901), Rural Development Administration, Republic of Korea.

Conflict of Interest

The authors declare no conflicts of interest.

References

1. **Anugwa FOI, Varel VH, Dickson JS, Pond WG, Krook LP.** Effects of dietary fiber and protein concentration on growth, feed efficiency, visceral organ weights and large intestine microbial populations of swine. J Nutr 1989, **119**, 879-886.
2. **Bederska-Łojewska D, Pieszka M.** Modulating gastrointestinal Microflora of pigs through nutrition using feed additives. Ann Anim Sci 2011, **11**, 333-335.
3. **Begley M, Gahan CG, Hill C.** The interaction between bacteria and bile. FEMS Microbiol Rev 2005, **29**, 625-651.
4. **Clinical and Laboratory Standards Institute.** Performance Standards for Antimicrobial Susceptibility Testing. Fifteenth informational supplement M100-S15. CLSI, Wayne, 2005.
5. **England LS, Lee H, Trevors JT.** Bacterial survival in soil: effect of clays and protozoa. Soil Biol Biochem 1993, **25**, 525-531.
6. **Forestier C, De Champs C, Vatoux C, Joly B.** Probiotic activities of *Lactobacillus casei rhamnosus*: in vitro adherence to intestinal cells and antimicrobial properties. Res Microbiol 2001, **152**, 167-173.
7. **Franz CMAP, Van Belkum MJ, Holzapfel WH, Abriouel H, Gálvez A.** Diversity of enterococcal bacteriocins and their grouping in a new classification scheme. FEMS Microbiol Rev 2007, **31**, 293-310.
8. **Gueimonde M, Sánchez B, de Los Reyes-Gavilán CG, Margolles A.** Antibiotic resistance in probiotic bacteria. Front Microbiol 2013, **4**, 202.
9. **Kim EY, Kim YH, Rhee MH, Song JC, Lee KW, Kim KS, Lee SP, Lee IS, Park SC.** Selection of *Lactobacillus* sp. PSC101 that produces active dietary enzymes such as amylase, lipase, phytase and protease in pigs. J Gen Appl Microbiol 2007, **53**, 111-117.
10. **Lee JS, Awji EG, Lee SJ, Tassew DD, Park YB, Park KS, Kim MK, Kim B, Park SC.** Effect of *Lactobacillus plantarum* CJLP243 on the growth performance and cytokine response of weaning pigs challenged with enterotoxigenic *Escherichia coli*. J Anim Sci 2012, **90**, 3709-3717.
11. **Longland AC, Low AG, Quelch DB, Bray SP.** Adaptation to the digestion of non-starch polysaccharide in growing pigs fed on cereal or semi-purified basal diets. Br J Nutr 1993, **70**, 557-566.
12. **Molist F, de Segura AG, Gasa J, Hermes RG, Manzanill EG, Anguita M, Pérez JF.** Effects of the insoluble and soluble dietary fibre on the physicochemical properties of digesta and the microbial activity in early weaned piglets. Anim Feed Sci Technol 2009, **149**, 346-353.
13. **Nueno-Palop C, Narbad A.** Probiotic assessment of *Enterococcus faecalis* CP58 isolated from human gut. Int J Food Microbiol 2011, **28**, 390-394.
14. **Pesenti-Barili B, Ferdani E, Mosti M, Degli-Innocenti F.** Survival of *Agrobacterium radiobacter* K84 on various carriers for crown gall control. Appl Environ Microbiol 1991, **57**, 2047-2051.
15. **Varel VH, Pond WG.** Enumeration and activity of cellulolytic bacteria from gestating swine fed various levels of dietary fiber. Appl Environ Microbiol 1985, **49**, 858-862.
16. **Wellinghausen N, Chatterjee I, Berger A, Niederfuehr A, Proctor RA, Kahl BC.** Characterization of clinical *Enterococcus faecalis* small-colony variants. J Clin Microbiol 2009, **47**, 2802-2811.