Antagonistic Activity of Polyfermenticin SCD Against *Helicobacter pylori* KCTC 2948

KIM, SEONG-MI, KWANG-HO LEE, NA-KYOUNG LEE, CHEON-JEI KIM[1], CHANG-HAN KIM[1], AND HYUN-DONG PAIK[1]*

Division of Life Sciences, Kyungnam University, Masan 631-701, Korea
[1]*Division of Animal Life Science, Konkuk University, Seoul 143-701, Korea*

Abstract *Bacillus polyfermenticus* SCD and polyfermenticin SCD, named tentatively as the bacteriocin produced by *B. polyfermenticus* SCD, showed antimicrobial activity against *Helicobacter pylori* KCTC 2948 growth. When crude polyfermenticin SCD was added to the growing *H. pylori* cells, viable cell numbers were reduced, indicating antimicrobial action. The antimicrobial effect was increased remarkably at higher concentrations of polyfermenticin SCD and longer exposure. Morphological changes were observed in the bacteriocin-treated cells; in the exponential phase, they appeared as shrunken rods, while in the stationary phase, they showed coccoid forms.

Key words: Bacteriocin, probiotics, *Bacillus polyfermenticus* SCD, polyfermenticin SCD, antimicrobial activity, *Helicobacter pylori*

Helicobacter pylori, a Gram-negative spiral bacterium, was first isolated from a patient with chronic gastritis by Warren & Marshall in 1983. Since then, much accumulated evidence shows a close relationship between gastroduodenal disease and *H. pylori* [8]. Eradication of *H. pylori* by combined treatment with antibiotics and antacids has been followed by a reduced rate of peptic ulcer recurrence and a steady resolution of underlying gastritis [1, 3, 9, 13]. The current therapies for the eradication of *H. pylori* infection require combinations of three or four medications, for example, amoxicillin as an antibiotic, omeprazol or metronidazole as proton pump inhibitors, and bismuth compounds. But, such antibiotic based therapies have some limitations, which include poor compliance, side effects, and antimicrobial resistance [2, 5].

Probiotics are living microorganisms which, when ingested, exert health benefits beyond general nutrition. These organisms alter the composition of the gastrointestinal flora by producing lactic acid, bacteriocins, and antimicrobial peptides, which inhibit pathogens. Bacterial pathogens inhibited by probiotics include *E. coli*, *Streptococcus*, *Clostridium*, *Bacteroides*, and *Salmonella*. Their additional benefits include production of mucosal micronutrients, elimination of toxins, and reduction of fecal ammonia, which is toxic to mucosa [12].

Bacillus polyfermenticus SCD, which is commonly referred to as Bisroot strain, an endospore-forming rod, is commercially available in Korea and Japan. It produces a variety of enzymes which lyse pathogenic strains like typhoid *Bacillus*, paratyphoid *Bacillus*, *Shigella*, and *Cholera*. The uptake of *B. polyfermenticus* SCD can enhance the appetite and promote digestion in humans by serving as a source of vitamin B_1 and B_2 and by protecting against nonoral infections and oral immunization [6, 16, 18]. Polyfermenticin SCD was named tentatively after the organism, *B. polyfermenticus* SCD, from which it was identified. This bacteriocin is a protein or protein complexes that has shown bactericidal activity against all *Bacillus* strains tested, as well as *Staphylococcus aureus* KCCM 32359, *Clostridium perfringenes* ATCC 3624, and *Micrococcus flavus* ATCC 10240 [7]. The purpose of this study was to evaluate the antimicrobial activity of polyfermenticin SCD against *H. pylori* KCTC 2948.

MATERIALS AND METHODS

Bacterial Strains and Media

Producer strain *B. polyfermenticus* SCD and indicator strain *H. pylori* KCTC 2948 were obtained from the R&D center of Binex Inc., Ltd. and the Korean Collection for

*Corresponding author
Phone: 82-2-2049-6011; Fax: 82-2-455-1044;
E-mail: hdpaik@konkuk.ac.kr

Type Cultures (KCTC), respectively. Working cultures were propagated in tryptic soy broth (TSB; Difco, Detroit, U.S.A.) with shaking at 30°C. The indicator organisms were placed on an agar plate containing a brain heart infusion (BHI; Difco) and 10% calf serum bovine (CSB; Sigma Chemical Co., St. Louis, U.S.A.), and grown in a 10% CO_2 incubator at 37°C [4, 11].

Production of Polyfermenticin SCD

Polyfermenticin SCD production was performed in a 5-l jar fermenter (3-l working volume; Korea Fermenter Co., Korea) in a TSB medium. *B. polyfermenticus* SCD was initially inoculated into 60 ml of sterile TSB and the seed culture (2%, v/v) obtained was transferred to a 5-l jar fermenter. The temperature was controlled at 30°C and the pH was maintained at 7.0±0.1 by adding 3 N H_2SO_4 or 3 N NaOH. The agitation speed was set at 500 rpm and the aeration rate at 1 vvm. Antifoam agent (silicone oil) was added automatically whenever necessary. Samples were aseptically removed at intervals over a 7-h period to determine cell growth and bacteriocin activity. Cell growth was monitored spectrophotometrically and the bacteriocin activity of the culture broth was determined as previously described [7].

Preparation of Crude Polyfermenticin SCD

Culture broth from the jar fermenter was centrifuged at 8,000 ×g for 20 min at 4°C and the supernatant was filter-sterilized through 0.22 µm cellulose acetate. Crude polyfermenticin SCD was obtained as described previously [14, 17].

Detection of Antimicrobial Activity

B. polyfermenticus SCD and polyfermenticin SCD were examined for antimicrobial activity against indicator organisms by the spot-on-lawn method [4]. In order to confirm that observed inhibition was due to the bacteriocin rather than some other inhibitor, such as acetic or organic acids, crude polyfermenticin SCD was treated for 3 h with proteinase K (Sigma, St. Louis, U.S.A.) at a final concentration of 1 mg/ml. Proteinase K-untreated polyfermenticin SCD and enzyme solution served as controls [7].

Cells from the exponential phase and stationary phase of *H. pylori* KCTC 2948 were suspended in 100 mM phosphate buffer (pH 7.0). The test was carried out at 37°C by adding 400 AU/ml or 1,000 AU/ml of crude polyfermenticin SCD. At various times, viable cell numbers (CFU/ml) were determined on BHI agar plates containing 10% CSB by the standard plate counting method [15].

Scanning Electron Microscopy

Morphological changes of *H. pylori* KCTC 2948 by crude polyfermenticin SCD were investigated by scanning electron microscopy (S-4200 FEG-SEM, Hitachi, Japan). The

following samples were prepared: a control without polyfermenticin SCD treatment, and exponential phase cells and stationary phase cells treated with 400 AU/ml of polyfermenticin SCD for 10 h. Samples were fixed in 25% (v/v) glutaraldehyde at 4°C for 12 h, and washed three times in 100 mM phosphate buffer (pH 7.0). They were dehydrated by passing through a graded ethanol series [60, 70, 80, 90, 95, and 100% (v/v)], and ion-spotter-coated with 200 nm gold in a vacuum evaporator. The SEM was operated at an accelerating voltage of 15.

RESULTS AND DISCUSSION

Antimicrobial Activity Against *H. pylori* KCTC 2948

The growth and adhesion of *H. pylori* was inhibited by egg yolk antibodies, Chinese tea, and garlic oil [5, 10, 19]. The antagonistic activity of *B. polyfermenticus* SCD against *H. pylori* KCTC 2948 was found to be a cause of bacteriocin and other inhibitory substance(s) including organic acids. Treatment with proteinase K destroys polyfermenticin
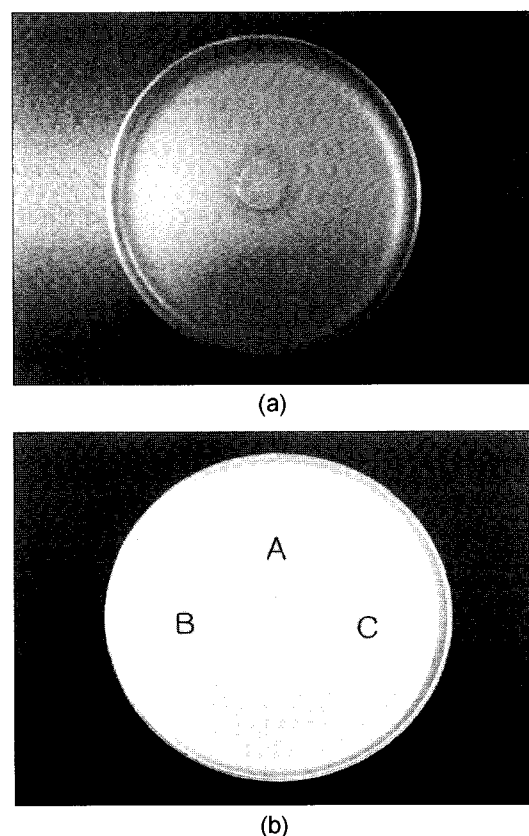

(a)

(b)

Fig. 1. Growth inhibition of *H. pylori* KCTC 2948 by (a) *B. polyfermenticus* SCD and (b) polyfermenticin SCD using the spot-on-lawn method.
A, Crude polyfermenticin SCD; B, Proteinase K-treated polyfermenticin SCD; C, Proteinase K.
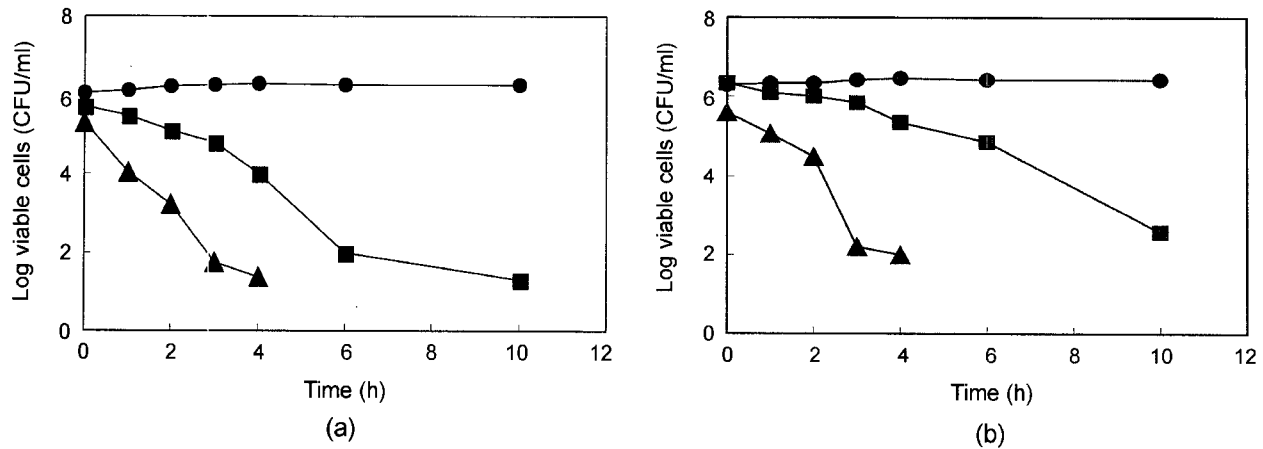
Fig. 2. Antimicrobial activity of crude polyfermenticin SCD against *H. pylori* KCTC 2948 at the exponential phase (a) and the stationary phase (b).
●, Control (0 AU/ml); ■, Polyfermenticin SCD (400 AU/ml); ▲, Polyfermenticin SCD (1,000 AU/ml).

SCD activity [7]. Therefore, 10 μl of *B. polyfermenticus* SCD, crude polyfermenticin SCD, proteinase K-treated polyfermenticin SCD, and enzyme solution were spotted onto plates, which had been overlaid with soft agar

containing *H. pylori* KCTC 2948. As shown in Fig. 1, *B. polyfermenticus* SCD and crude polyfermenticin SCD showed a clear zone against *H. pylori* KCTC 2948. However, proteinase K-treated polyfermenticin SCD and
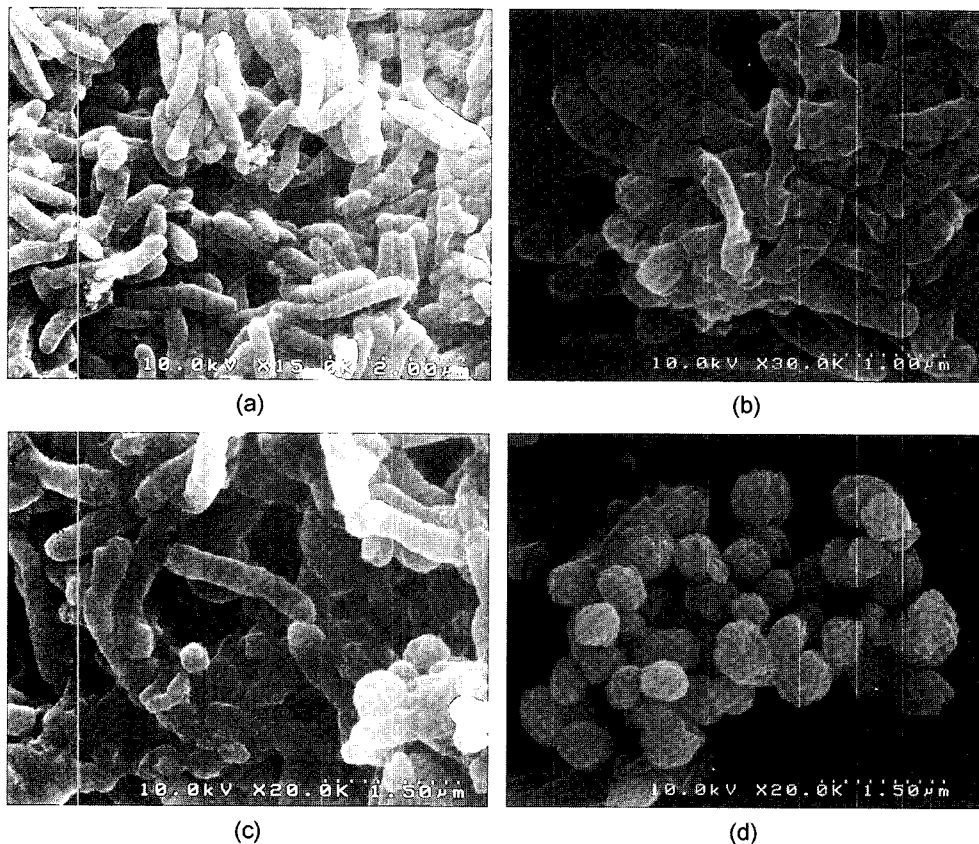

Fig. 3. Scanning electron microscopic (SEM) observations of *H. pylori* KCTC 2948 treated with polyfermenticin SCD.
(a) Exponential phase cells, (b) exponential phase cells treated with polyfermenticin SCD, (c) stationary phase cells, (d) stationary phase cells treated with polyfermenticin SCD.

enzyme solution caused a complete loss of inhibitory activity, indicating that the antagonistic activity of *B. polyfermenticus* SCD against *H. pylori* KCTC 2948 was due to bacteriocin.

Effect of the Growth Phase on Anti-*Helicobacter pylori* Activity

To examine the antimicrobial activity of *H. pylori* KCTC 2948 in the exponential and stationary phases, viable cells of *H. pylori* KCTC 2948 with and without polyfermenticin SCD were determined. The number of viable cells was decreased after exposing *H. pylori* cells to the bacteriocin (Fig. 2). When the crude polyfermenticin SCD was added to the exponential and stationary phase cultures of *H. pylori* KCTC 2948 at 400 AU/ml, a reduction of approximately 4.4 to 3.8 log CFU/ml of *H. pylori* was observed in 10 h, and when 1,000 AU/ml of polyfermenticin SCD was added, this reduced to below 2.0 log CFU/ml at 4 h.

Morphological Changes Induced by Polyfermenticin SCD

Morphological changes of *H. pylori* KCTC 2948 cells with and without crude polyfermenticin SCD were investigated by FEG-SEM. Cells without bacteriocin showed rod shapes in the exponential and stationary phases (Fig. 3a, 3c). In addition, the cells treated with 400 AU/ml of crude polyfermenticin SCD showed shrunken rod shapes in the exponential phase (Fig. 3b), but perfect coccoid forms in the stationary phase (Fig. 3d).

The morphological change of *H. pylori* from bacillary to coccoid can occur in response to environmental stress, such as nutrient depletion, accumulation of toxic metabolites, pH change, or exposure to an antimicrobial agent [2, 13, 20]. The coccoid forms are unculturable by applying the conventional techniques, but they may still be viable, thus posing a risk of infection. Rod-shaped *H. pylori* is believed to be responsible for chronic infections of the stomach, while the coccoid form has been implicated in transmission. As a result, the morphological changes imply potential of polyfermenticin SCD for *H. pylori* therapy.

In conclusion, the inhibitory effect and mode of action of polyfermenticin SCD against *H. pylori* KCTC 2948 were demonstrated in this study. When polyfermenticin SCD was added to the growing indicator cells, viable cell numbers were decreased, demonstrating its bactericidal mode of action. Moreover, this antimicrobial effect was enhanced at higher polyfermenticin SCD concentrations and increased exposure times. Morphological changes were observed when the cells were treated with the bacteriocin in the exponential phase as shrunken rod shapes, and the cells in the stationary phase were found to be coccoid in shape. These characteristics would imply a promising approach to *H. pylori* therapy.

Acknowledgment

References

1. Graham, D. Y., G. M. Lew, P. D. Klein, D. G. Evans, Z. A. Saeed, and H. M. Malaty. 1992. Effect of treatment of *Helicobacter pylori* infection on the long term recurrence of gastric or duodenal ulcer - a randomize, controlled study. *Ann. Intern. Med.* **116:** 705–708.
2. Harris, A. 1997. Treatment of *Helicobacter pylori*. *Drugs Today* **33:** 59–66.
3. Kim, J.-M., J.-E. Shin, M. J. Han, S.-H. Park, and D.-H. Kim. 2003. Inhibitory effect of Ginseng saponins and polysaccharides on infection and vacuolation of *Helicobacter pylori*. *J. Microbiol. Biotechnol.* **13:** 706–709.
4. Kim, M.-R., S.-K. Yun, W.-J. Lim, B.-S. Hong, and S.-Y. Hwang. 1999. Synergistic inhibition of membrane ATPase and cell growth of *Helicobacter pylori* by ATPase inhibitors. *J. Microbiol. Biotechnol.* **9:** 414–421.
5. Koo, J.-K., C.-H. Kim, and T.-B. Choe. 1999. Inhibition of growth and adhesion of *Helicobacter pylori* using egg yolk antibodies. *Biotechnol. Bioprocess Eng.* **4:** 219–223.
6. Lee, K.-H. and H.-D. Paik. 1999. Gastric and bile acid tolerance on commercial probiotic products. *Environ. Res.* **22:** 73–78.
7. Lee, K.-H., K.-D. Jun, W.-S. Kim, and H.-D. Paik. 2001. Partial characterization of polyfermenticin SCD, a newly identified bacteriocin of *Bacillus polyfermenticus*. *Lett. Appl. Microbiol.* **32:** 146–151.
8. Marshall, B. J. and S. R. Langton. 1986. Urea hydrolysis in patients with *Campylobacter pyloridis* infection. *Lancet* **1:** 965–966.
9. Marshall, B. J., C. S. Goodwin, J. R. Warren, R. Murray, E. D. Blincow, S. J. Blackbourn, M. Phillips, T. E. Waters, and C. R. Sanderson. 1998. Prospective double-blind trial of duodenal ulcer relapse after eradication of *Campylobacter pylori*. *Lancet* **2:** 1437–1442.
10. McNulty, C. A. M., M. P. Wilson, W. Havinga, B. Johnston, E. A. O'Gara, and D. J. Maslin. 2001. A pilot study to determine the effectiveness of garlic oil capsules in the treatment of dyspeptic patients with *Helicobacter pylori*. *Helicobacter* **6:** 249–253.
11. Nam, G.-J., S.-W. Yeon, N.-S. Paek, T.-H. Kim, Y. H. Kim, C. J. Kim, and K. W. Kim. 1998. Isolation and structural determination of anti-*Helicobacter pylori* compound from Fungus 60686. *Kor. J. Appl. Microbiol. Biotechnol.* **26:** 137–142.
12. O'Sullivan, G. C. 2001. Probiotics. *Br. J. Surgery* **88:** 161–162.
13. Peterson, W. L. 1991. *Helicobacter pylori* and peptic ulcer disease. *N. Engl. J. Med.* **324:** 1043–1048.
14. Paik, H.-D. and B. A. Glatz. 1995. Purification and partial amino acid sequences of propionicin PLG-1, a bacteriocin

produced by *Propionibacterium thoenii* P127. *Lait* **75**: 367–377.

15. Paik, H.-D., S.-S. Bae, S.-H. Park, and J.-G. Pan. 1997. Identification and partial characterization of tochicin, a bacteriocin produced by *Bacillus thuringiensis* subsp. *tochigiensis*. *J. Ind. Microbiol. Biotechnol.* **19**: 294–298.

16. Paik, H.-D., N.-K. Lee, K.-H. Lee, Y.-I. Hwang, and J.-G. Pan. 2000. Identification and partial characterization of cerein BS229, a bacteriocin produced by *Bacillus cereus* BS229. *J. Microbiol. Biotechnol.* **10**: 195–200.

17. Park, H.-S., S.-H. Lee, and T.-B. Uhm. 1998. Selection of microorganism for probiotics and their characterization. *J. Kor. Soc. Food Sci. Nutr.* **27**: 433–440.

18. Park, K.-Y., H.-Y. Jung, K.-L. Woo, K.-D. Jun, J.-S. Kang, and H.-D. Paik. 2002. Effects of *Bacillus polyfermenticus* SCD administration on fecal microflora and putrefactive metabolites in healthy adults. *J. Microbiol. Biotechnol.* **12**: 657–663.

19. Yee, Y.-K., M. W.-L. Koo, and M.-L. Szeto. 2002. Chinese tea consumption and low risk of *Helicobacter* infection. *J. Gastro. Hepatol.* **17**: 552–555.

20. Young, K. A., P. P. Allaker, and J. M. Hardie. 2001. Morphological analysis of *Helicobacter pylori* from gastric biopsies and dental plaque by scanning electron microscopy. *Oral Microbiol. Immunol.* **16**: 178–181.