Walailak J Sci & Tech 2008; 5(2): 161-171.

A Comparison of Two Methods Used for Measuring the Antagonistic Activity of *Bacillus* Species

Monthon LERTCANAWANICHAKUL and Songtham SAWANGNOP

School of Allied Health Sciences and Public Health, Walailak University, Nakhon Si Thammarat 80161, Thailand

(E-mail: lmonthon@wu.ac.th)

ABSTRACT

In this study, we have aimed to determine antagonistic effects of various *Bacillus* against representatives of Gram (+) and Gram (–) bacteria, (*Staphylococcus aureus* TISTR 517 and *Escherichia coli* TISTR 887) with a comparison between the cross streak method and agar well diffusion method. Both methods used in the experiment gave better inhibition results on the *S. aureus* TISTR 517 compared to the *E. coli* TISTR 887. Interestingly, in the case of the cross streak method, both indicator bacteria were clearly inhibited in their growth by the *Bacillus* species used in this study. The cross streak method was suitable for a preliminary assessment of the antagonistic effects of *Bacillus* species.

Keywords: Antagonistic activity, cross streak method, well diffusion method

INTRODUCTION

Probiotics are commonly used as viable microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial "balance" [1-3], improving nutrition and growth, and prevention of various gastrointestinal disorders. Probiotics used in animals include *Lactobacillus*, *Bifidobacterium*, *Bacillus*, *Streptococcus*, *Pediococcus*, *Enterococcus*, and yeasts such as *Saccharomyces cerevisiae* and *S. boulardii* [3]. Moreover, probiotic containing products are available for human nutrition [4] and in some countries probiotics are taken as prophylactic and therapeutic agents [5]. The emphasis has been placed on the selection and preparation of probiotic strains. Most work has focused on *Lactobacillus* spp. [6], *Bifidobacterium* spp. [7] and *Enterococcus* spp. [8]. Several strains of *Bacillus* have also shown promising characteristics [9,10]. Products containing endospores of members of the genus *Bacillus* (in single doses of up to 10^9 spores/g or 10^9 spores/ml) are used commercially as probiotics, and they offer some advantages over the more common *Lactobacillus* products in that they are easily cultured in "bulk" and can be stored indefinitely in a desiccate form [5], and still posses the capacity to sporulate [11].

Probiotics have antagonistic effects on various microorganisms, such as, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas* spp., *Proteus vulgaris*, *Salmonella typhimurium* [12-14]. There are many techniques for detecting antimicrobial activity. Some of them are based on dilution of antimicrobial agents in broth but most techniques are based on the diffusion through solid or semi-solid culture media to inhibit the growth of sensitive microorganisms [15-24]. The purposes of this study are to compare the cross streak method and well diffusion method to determine the most reliable method for the detection of antimicrobial activity and to show the effect of antimicrobial agents' inhibitory activity against *S. aureus* TISTR 517 as representative gram-positive bacteria and *E. coli* TISTR 887 as representative gram-negative bacteria.

MATERIALS AND METHODS

Bacterial Strains and Culture Media

The strains used in this study are listed in **Table 1**. All *Bacillus* species were cultured under shake tube conditions in 5 ml of Luria-Bertani (LB: Scharlau) broth for 24 h at 37 °C and 150 rpm before being transferred to LB agar plates incubated at 37 °C for 24 h and then stored in the refrigerator. Indicator microorganisms were maintained on LB agar at 4 °C. All strains were maintained in the LB broth containing 15 % glycerol at –80 °C.

Table 1 Reference of bacterial strains.

***Bacillus* species**	**Source or Reference**
Brevibacillus laterosporus SA14	[25]
Bacillus megaterium BA6	[26]
Brevibacillus non-reactive BA7	[26]
Geobacillus thermoglucosidasius BA16	[26]
Indicator bacteria	
Staphylococcus aureus TISTR 517 *Escherichia coli* TISTR 887	Thailand Institute of Scientific and Technological Research, Bangkok, Thailand.

Preparation of Culture Broth

Bacillus species were grown at 37 °C for 18 h in LB broth. The 2 % seed culture, correlated to standard McFarland No.0.5, was transferred to a 250 ml flask with 150 ml of LB broth. The experiment was carried out in duplicate. The culture flask was incubated at 37 °C for 13 days at 150 rpm. After every 24 h, 1 ml of the culture was drawn from each flask and centrifuged at 14,000 g for 20 min to pellet the cells. The culture broth (CB) was collected and used for the antagonistic activity assay by means of agar well diffusion.

Preparation of Washed Cell

Washed cells were prepared as described by Kivanc [20], with some modifications. Twenty four hour cultured indicator bacteria were centrifuged at 14,000 g for 15 min and cells were collected and supernatants were discarded. Cells were washed twice with sterile 0.85 % NaCl and were approximately adjusted to 10^8 cells/ml, using McFarland No.0.5 as a standard solution.

Cross Streak Method

LB agar plates were prepared and inoculated with various species of *Bacillus* by a single streak of inoculum in the center of the petri dish (plate). After 2 days of incubation at 37 °C the plates were seeded with indicator bacteria by a single streak at a 90° angle to the *Bacillus* species. The microbial interactions were analyzed by the observation of the size of the inhibition zone [27].

Agar Well Diffusion Method

Both indicator bacteria were cultured on LB broth and incubated at 37 °C for 24 h. LB agar (25 ml) was poured into each sterilised petri dish (90 mm in diameter). Suspensions (100 µl), equivalent to standard McFarland No. 0.5, of indicator bacteria cultured for 24 h were spread on the plates, and wells 10 mm in diameter were punched in the agar with a sterile tip. The CB(s) of each *Bacillus* culture was prepared as

described above. Each CB (100 µl) was directly filled into the wells of agar plates inoculated with indicator bacteria. The plates were incubated for 24 h at 37 °C, and the diameter of the inhibition zone was measured in millimeters.

RESULTS

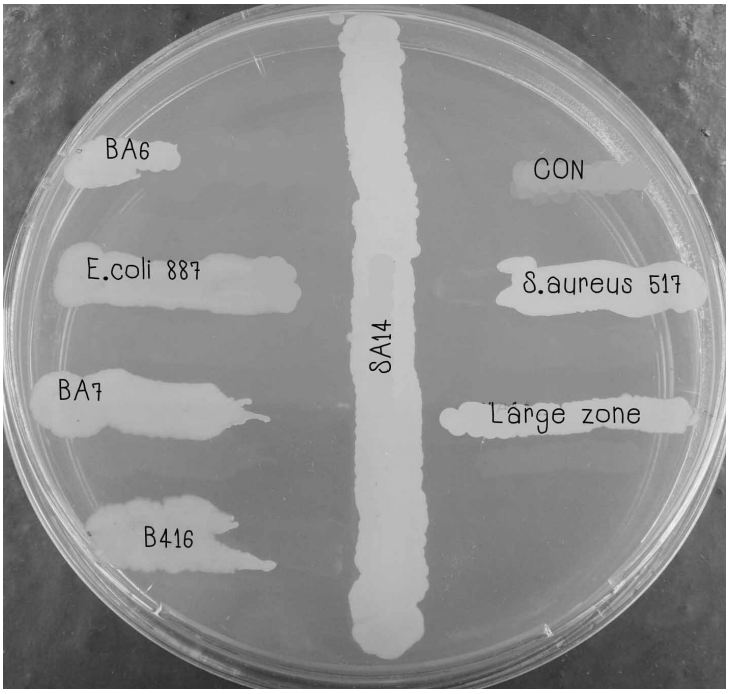
Four *Bacillus* species were tested against 2 indicator bacteria (**Table 1**). The results of the cross streak method showed that all *Bacillus* species exhibited inhibitory activity after 2 days of incubation. Interestingly, 1-day old *Brevibacillus laterosporus* SA14 could exhibit antibacterial activity when tested by the cross streak method (data not shown). The results of the agar well diffusion method in determining the antibacterial activity of *Bacillus* species on indicator bacteria are listed in **Table 2**. Most *Bacillus* species exhibited inhibitory activity after 72 h of incubation and remained up to the 5th day. In both the cross streak and agar well diffusion methods, *S. aureus* TISTR 517 was more sensitive than *E. coli* TISTR 887.

The results obtained from agar well diffusion showed that *E. coli* TISTR 887 was more resistant than *S. aureus* TISTR 517 to *Bacillus* species, however in the cross streak method this organism had inhibited growth. Similarly, for example, *Brevibacillus laterosporus* SA14 possessed inhibitory activity against *E. coli* TISTR 887 in the cross streak method, but it was less active in the agar well diffusion method as shown in **Figure 1**. The results showed that the initial antagonistic activity was observed on 1-day old *Brevibacillus laterosporus* SA14 in the cross streak method (**Figure 1a**). In contrast, the initial antagonistic activity (inhibition zone) was observed around well of CB prepared from the 3-day old *Brevibacillus laterosporus* SA14 in the agar the well diffusion method (4 in **Figure 1b** and **Table 2**).

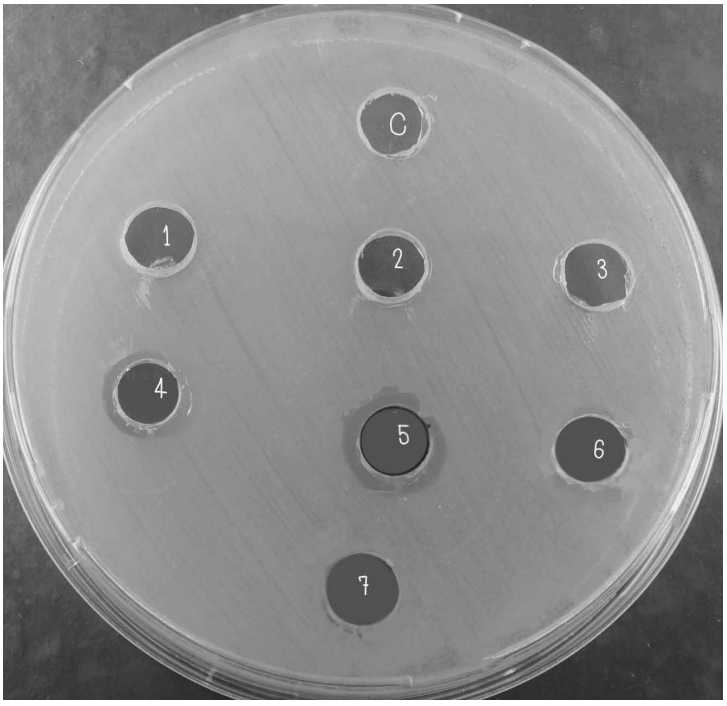
Table 2 Antagonistic effect of *Bacillus* species against indicator microorganisms by the agar well diffusion method.

***Bacillus* species**[1]	**Indicator bacteria**[2]	**Diameter of inhibition zone (mm)**												
		Day1	**Day2**	**Day3**	**Day4**	**Day5**	**Day6**	**Day7**	**Day8**	**Day9**	**Day10**	**Day11**	**Day12**	**Day13**
SA14	S	0	3	6	4	10	12	11	10	10	11	6	4	0
	E	0	0	1	2	0	0	0	0	0	0	0	0	0
BA6	S	0	0	6	7	6	7	7	4	3	2	2	3	2
	E	0	0	2	2	1	0	0	0	0	0	0	0	0
BA7	S	0	0	10	8	7	6	5	5	5	4	2	3	2
	E	0	0	3	1	0	0	0	0	0	0	0	0	0
BA16	S	0	0	1	2	1	0	0	0	0	0	0	0	0
	E	0	0	0	0	0	0	0	0	0	0	0	0	0

[1]SA14, *Brevibacillus laterosporus* SA14; BA6, *Bacillus megaterium* BA6; BA7, *Brevibacillus* non-reactive BA7; BA16, *Geobacillus thermoglucosidasius* BA16.
[2]S, *Staphylococcus aureus* TISTR 517; E, *Escherichia coli* TISTR 887.


BA6
CON
E.coli 887
S.aureus 517
SA14
BA7
Large zone
B416

(a)


C
1
2
3
4
5
6
7

(b)

Figure 1 Example of antagonistic activity assay of *Bacillus* species. The cross streak method (a) and the agar well diffusion method (b). (a); SA14, *Brevibacillus laterosporus* SA14; BA6, *Bacillus megaterium* BA6; BA7, *Brevibacillus* non-reactive BA7; BA16, *Geobacillus thermoglucosidasius* BA16; S.aureus 517, *Staphylococcus aureus* TISTR 517; E.coli 887, *Escherichia coli* TISTR 887; CON, sensitive strain; large zone, resistant strain. (b); C, medium broth; 1-7, culture broth (CB) from 0-day to 6-day old *Brevibacillus laterosposus* SA14, respectively; *E. coli* TISTR 887 was used as the indicator bacterium. (see more details in **MATERIALS AND METHODS**).

DISCUSSION AND CONCLUSION

Various strains of *Bacillus* species displayed antimicrobial activity against *Pseudomonas aeruginosa*, *P. fluorescens* RSKK 380, *B. thuringiensis* RSKK 380, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *Micrococcus luteus*, *M. flavus*, *Yersinia enterocolitica*, *Bacillus subtilis*, *B. megaterium*, methicillin-resistant *S. aureus*, and *Candida albicans* [25, 28-30].

We determined the antagonistic effects of various *Bacillus* against *S. aureus* TISTR 517 (Gram +) and *E. coli* TISTR 887 (Gram −) in a comparison of the cross streak and agar well diffusion methods. Both antibacterial activity methods showed that the Gram (+) bacterium, *S. aureus*, was the most sensitive indicator bacteria. The *Bacillus* strains were active most against Gram-positive but not Gram-negative bacteria [30,31]. Although, the *E. coli* TISTR 887 was the most resistant indicator bacterium, its growth was inhibited when using the cross streak method. The use of the cross streak method in this experiment resulted in higher inhibition results than the use of the agar well diffusion method. As a result, the inhibitory activity on indicator bacteria by the cross streak method is seen as better, but it could be because all metabolites and bacteriocin are present and being produced during the assay period. But the agar well diffusion method, CB (supernatants) of *Bacillus* species were used, and conditions for the growing bacteria and/or the preparation of CB may result in a decrease of other metabolites inhibitory activity. So, the inhibition zone that was seen around the wells may be dependent solely on bacteriocin activity.

Furthermore, the substances *Bacillus* species secreted might be in the log- or late log-phase of growth. The CB which was collected in the stationary-phase of growth (according to the agar well diffusion method) did not give an inhibition zone, whereas the cross streak method showed inhibition results. The metabolite might be in the log-phase and destroyed by other metabolites [32] or used as a precursor to produce other metabolites, causing no inhibition zone around the wells of CB prepared from 5-day and 6-day old culture when tested by the agar well diffusion method (6 and 7 in **Figure 1b**). In the cross streak environment, the *Bacillus* had to compete with other microorganisms, then the *Bacillus* produce the metabolites and did not use them as precursors resulting in enough metabolites remaining in the agar.

In conclusion, in antimicrobial activity research the cross streak method is a practical and suitable technique. However in bacteriocin investigations, the cross streak method should be controlled with the agar well diffusion method. The antimicrobial compounds, produced by *Bacillus* cultures, have a great potential for controlling the growth of microorganisms [5, 9-11, 25, 28-30].

ACKNOWLEDGEMENTS

This work was supported by a grant (WU51109) from Walailak University. The help of the staff from the laboratories at Walailak University are gratefully acknowledged.

REFERENCES

[1] R Fuller. Probiotics in man and animals. *J. Appl. Bacteriol.* 1989; **66**, 365-78.
[2] R Fuller. Probiotics in human medicine. *Gut* 1991; **32**, 439-42.
[3] R Fuller. *Probiotics: The Scientific Basis*. Chapman and Hall, London, 1992, p. 1-8, 209-57.
[4] I Rowland. Probiotics and benefits to human health-the evidence in favor. *Environ. Microbiol.* 1999; **1**, 375-82.
[5] P Mazza. The use of *Bacillus subtilis* as antidiarrhoeal microorganisms. *Boll. Chim. Farm.* 1994; **133**, 3-18.
[6] AM Kabir, Y Aiba, A Takagi, S Kamiya, T Miwa and Y Koga. Prevention of *Helicobater pylori* infection by Lactobacilli in a gnotobiotic murine model. *Gut* 1997; **41**, 49-55.
[7] J Singh, A Rivenson, M Tomita, S Shimamura, N Ishibashi and BS Reddy. *Bifidobacterium longum*, a lactic acid producing intestinal bacterium inhibits colon cancer and modulates the intermediate biomarkers of colon carcinogenesis. *Carcinogenesis* 1997; **4**, 833-41.
[8] S Salminen, E Isolauri and E Salminen. Clinical uses of probiotics for stabilizing the gut mucosal barrier: successful strains and future challenges. *Antonie van Leeuwenhoek* 1998; **70**, 347-58.
[9] V Cavazzoni and A Adami. Biomass production, preservation and characteristic of a strain of *Bacillus coagulans* usable as probiotic. *Microbiologie-Aliments-Nutrition* 1993; **11**, 93-100.
[10] I Kumprecht and P Zobac. Continuous application of probiotics based on *Saccharomyces cerevisiae* var. *elipsoideus* and *Bacillus* C.I.P. 5832 in the nutrition of chicken broilers. *Anim. Sci.* 1996; **41**, 311-6.
[11] B Hyronimus, CL Marrec, AH Sassi and A Deschamps. Acid and bile tolerance of spore-forming lactic acid bacteria. *Int. J. Food Microbiol.* 2000; **61**, 193-7.

[12] JM Rodriguez, OJ Sobrino, MF Fernandez, PE Hernandez and B Sanz. Antimicrobial activity of lactic acid bacteria isolated from Spanish dry fermented sausages volume II. *In*: Proceedings of the 35th International Congress, Meat Sci. Tec., Copenhagen. 1989, p. 308-12.

[13] A Unluturk and F Turantas. Food Microbiology. Mengi Tan Press, Cinarli, Izmir. 1992, p. 605.

[14] GM Vignolo, F Suriani, APR Holdago and G Oliver. Antibacterial activity of *Lactobacillus* strains isolated from dry fermented sausages. *J. Appl. Bacteriol.* 1993; **75**, 344-9.

[15] EC Parente, C Brienza, M Moles and A Ricciardi. A Comparison of methods for the measurement of bacteriocin activity. *J. Microbiol. Meth.* 1995; **22**, 95-108.

[16] CB Lewus and TJ Montville. Detection of bacteriocins produced by lactic acid bacteria. *J. Microbiol. Meth.* 1991; **13**, 145-50.

[17] SR Spelheugh and SK Harlander. Inhibition of bacterial food-borne pathogens by bacteriocins from *Lactococcus lactis* and *Pediococcus pentosaceous*. *J. Food Protect.* 1989; **52**, 856-62.

[18] ML Stecchini, V Aquili, I Sarais and A Pitotti. Inhibition of *Listeria monocytogenes* by *Lactococcus lactis* subsp. *lactis* isolated from Italian raw ham. *J. Food Safety* 1992; **12**, 295-302.

[19] PK Sarkar and S Banerjee. Antibacterial activity of lactic acid bacterial isolates obtained from natural habitats. *J. Food Sci. Tec.* 1996; **33**, 231-3.

[20] M Kivanc. Antagonistic action of lactic cultures toward spoilage and pathogenic microorganisms in food. *Die Nahrung* 1990; **34**, 273-7.

[21] SR Pulusani, DR Rao and GR Sunki. Antibacterial activity of lactic cultures: partial purification and characterization of antimicrobial compound(s) produced by *Streptococcus thermophilus*. *J. Food Sci.* 1979; **44**, 575-8.

[22] HP Fleming, JL Etchells and RN Costilow. Microbial inhibition by an isolate of *Pediococcus* from cucumber brines. *Appl. Microbiol.* 1975; **30**, 1040-42.

[23] U Schillinger and FK Lucke. Antibacterial activity of *Lactobacillus saki* isolated from meat. *Appl. Environ. Microbiol.* 1989; **55**, 1901-06.

[24] AH Con, HY Gokalp and M Koya. Antagonistic effect on *Listeria monocytogenes* and *L. innocua* of a bacteriocin-like metabolite produced by lactic acid bacteria isolated from sucuk. *Meat Sci.* 2001; **59**, 437-41.

[25] A Choopan, K Nakbud, K Dawveerakul and M Lertcanawanichakul. Anti-methicillin resistant *Staphylococcus aureus* activity of *Brevibacillus laterosporus* strain SA14. *Walailak J. Sci. & Tech.* 2008; **5**, 47-56.

[26] K Santhong, S Naorungrote, P Bangrak, W Chunglok and M Lertcanawanichakul. Screening, identification and antibacterial activities of effective thermotolerant *Bacillus* spp. strains isolated from raw milk. *Walailak J. Sci. & Tech.* 2008; **5**, 39-46.

[27] MT Madigan, JM Martiko and J Parker. *Antibiotics: Isolation and Characterization*. *In*: MT Madigan (ed). Brock Biology of Microorganisms. 8th ed. Prentice-Hall International Inc., New Jersey, 1997, p. 440-2.

[28] B Aslim, N Saglam and Y Beyatli. Determination of some properties of *Bacillus* isolated from soil. *Turk. J. Biol.* 2002; **26**, 41-8.
[29] M Yilmaz, H Soran and Y Beyatli. Antimicrobial activities of some *Bacillus* spp. strains isolated from the soil. *Microbiol. Res.* 2006; **161**, 127-31.
[30] R Eltem and F Ucar. The determination of antimicrobial activity spectrum of 23 *Bacillus* strains isolated from Denizli-Acigol (Bitter Lake) which is soda lake (Na_2SO_4). *J. KUKEM* 1998; **21**, 57-64.
[31] M Morikawa, M Ito and T Imanaka. Isolation of a new surfactin producer *Bacillus pumilus* A-1, and cloning and nucleotide sequence of the regulator gene, *psf-1*. *J. Ferment. Bioeng.* 1992; **74**, 255-61.
[32] G Bhuvaneswari, P Padmanabhan, A Kapley and HJ Purohit. Study on *Staphylococcus aureus* Strain HPC-250 for associated antibacterial property. *Curr. Microbiol.* 2005; **51**, 287-91.

บทคัดย่อ

มณฑล เลิศคณาวนิชกุล และ ทรงธรรม สว่างนพ

การเปรียบเทียบวิธี 2 วิธีในการศึกษาฤทธิ์การเป็นปฏิปักษ์ของบาซิลลัส สปีชีส์

ได้ทำการศึกษาฤทธิ์ในการต้านสแตปฟิลโลคอคคัสออเรียส ทีไอเอสทีอาร์ 517 และเอชเชอริเชียคอไล ทีไอเอสทีอาร์ 887 ของบาซิลลัสสปีชีส์ด้วยวิธีการ 2 วิธีการ กล่าวคือ วิธีขีดเชื้อขวางเพลตและวิธีซึมผ่านของสารเข้าเนื้อวุ้น ปรากฏว่าบาซิลลัสสปีชีส์ที่นำมาทดสอบออกฤทธิ์ต้านสแตปฟิลโลคอคคัสออเรียส ทีไอเอสทีอาร์ 517 ได้ดีจากทั้ง 2 วิธีแต่ฤทธิ์ต้านเอชเชอริเชียคอไล ทีไอเอสทีอาร์ 887 จะเห็นได้ชัดเจนเมื่อทดสอบด้วยวิธีขีดเชื้อขวางเพลต ซึ่งอาจกล่าวได้ว่าวิธีขีดเชื้อขวางเพลตเป็นวิธีเบื้องต้นที่เหมาะสมนำมาใช้ศึกษาฤทธิ์ต้านจุลินทรีย์ของบาซิลลัสสปีชีส์

สำนักวิชาสหเวชศาสตร์และสาธารณสุขศาสตร์ มหาวิทยาลัยวลัยลักษณ์ อำเภอท่าศาลา จังหวัดนครศรีธรรมราช 80161