บทความวิจัย

การศึกษาเบื้องต้นของเชื้อแลคโตบาซิลลัสสายพันธุ์ไทยที่มีฤทธิ์ยับยั้งการเจริญของเชื้อก่อโรคในลำไส้

เธียร ธีระวรวงศ์

บทคัดย่อ

งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ต้านเชื้อของแลคโตบาซิลลัสสายพันธุ์ไทยจำนวน 227 ไอโซเลตต่อเชื้อก่อโรคในลำไส้ในหลอดทดลอง แลคโตบาซิลลัสถูกจุด (spot) ลงบนอาหารเลี้ยงเชื้อ Brain Heart Infusion agar ที่มีการเติม 20 mM กลูโคส และเททับ (overlay) ด้วยเชื้อก่อโรคในลำไส้ ผลการศึกษาพบว่าแลคโตบาซิลลัสจำนวน 78 ไอโซเลตจาก 227 ไอโซเลตสามารถยับยั้งเชื้อ *Shigella flexneri* หรือ *Vibrio cholerae* แลคโตบาซิลลัสทุกไอโซเลตไม่สามารถยับยั้งเชื้อ *Salmonella enterica* Typhimurium, enterohemorrhagic *Escherichia coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC) และสายพันธุ์ *E. coli* ที่เป็นเชื้อประจำถิ่น/ไม่ก่อโรค เป็นที่น่าสนใจว่าเมื่อจุดเชื้อ *L. fermentum* B66 อยู่จุดเดียว เชื้อจะไม่สามารถยับยั้ง *S. flexneri* และ *V. cholerae* ได้ แต่เมื่อจุดร่วมกับ *L. gasseri* B49, *L. plantarum* B64, *L. plantarum* B67 และ *L. mucosae* B79 เชื้อจะแสดงคุณสมบัติการเสริมฤทธิ์ซึ่งสามารถยับยั้งเชื้อก่อโรคได้ ยิ่งไปกว่านั้น *L. salivarius* T38 และ *L. salivarius* T70 เมื่อจุดร่วมกับ *L. casei* T20, *L. fermentum* T37, *L. salivarius* T39, *L. mucosae* T78 และ *L. reuteri* T36, *L. salivarius* T69, *L. salivarius* T71, *L. mucosae* T102 ตามลำดับ สามารถแสดงให้เห็นถึงขนาดของบริเวณใสที่ใหญ่กว่าในการยับยั้งเชื้อ *S. flexneri* และ *V. cholerae* เมื่อเทียบกับการจุดแลคโตบาซิลลัสเป็นเชื้อเดี่ยว อย่างไรก็ตาม เมื่อนำอาหารเหลวที่ผ่านการเลี้ยงเชื้อแลคโตบาซิลลัสมาทำให้เข้มข้นแล้วพบว่าไม่มีฤทธิ์ยับยั้งเชื้อก่อโรค โดยสรุปการศึกษานี้แสดงให้เห็นถึงแลคโตบาซิลลัสสายพันธุ์ไทยที่มีฤทธิ์ต้านและการเสริมฤทธิ์สามารถเพิ่มความสามารถในการยับยั้งเชื้อก่อโรคในลำไส้ซึ่งเป็นสาเหตุของโรคอุจจาระร่วงได้ เนื่องจากแลคโตบาซิลลัสไม่ได้มีการยับยั้ง *E. coli* ที่เป็นเชื้อประจำถิ่น ดังนั้น เชื้อแลคโตบาซิลลัสเหล่านี้จึงมีศักยภาพเป็นโพรไบโอติกป้องกันการติดเชื้อ *S. flexneri* หรือ *V. cholerae* ในลำไส้ได้

คำสำคัญ: แลคโตบาซิลลัส, ฤทธิ์ต้าน, การเสริมฤทธิ์, เชื้อก่อโรคในลำไส้

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Preliminary study of antibacterial activity of *Lactobacillus* Thai isolates against enteric pathogens

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ABSTRACT

The objective of this study was to determine the antibacterial activity of 227 *Lactobacillus* Thai isolates against various enteric pathogens *in vitro*. *Lactobacillus* spp. were spotted on Brain Heart Infusion agar supplemented with 20 mM glucose and overlaid by enteric pathogens. Seventy-eight of 227 isolates inhibited *Shigella flexneri* or *Vibrio cholerae*. All of the *Lactobacillus* isolates did not inhibit *Salmonella enterica* Typhimurium, enterohemorrhagic *Escherichia coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), and commensal/non-pathogenic *E. coli*. Interestingly, *L. fermentum* B66 alone did not inhibit *S. flexneri* and *V. cholerae*, but when spotted in conjunction with *L. gasseri* B49, *L. plantarum* B64, *L. plantarum* B67, and *L. mucosae* B79, it acquired the synergistic activity to inhibit the pathogens. In addition, *L. salivarius* T38 and *L. salivarius* T70 when spotted in conjunction with *L. casei* T20, *L. fermentum* T37, *L. salivarius* T39, *L. mucosae* T78, and *L. reuteri* T36, *L. salivarius* T69, *L. salivarius* T71, *L. mucosae* T102, respectively, indeed showed larger clear zones to *S. flexneri* and *V. cholerae* than the spot of each *Lactobacillus* alone. However, concentrated culture supernatants of these lactobacilli did not inhibit the pathogens. In conclusion, this study demonstrated that the *Lactobacillus* Thai isolates had both antibacterial and synergistic activities which could enhance the inhibitory effects on the enteric pathogens causing diarrheal disease. Since they did not inhibit commensal *E. coli*, these lactobacilli could be potential probiotics against *S. flexneri* or *V. cholerae* infection.

Keywords: *Lactobacillus*, antibacterial activity, synergistic activity, enteric pathogens

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Introduction

Diarrheal disease causing by enteric pathogens is problematic around the world. Particularly, in developing countries, high morbidity and mortality rate can occur commonly in infants and children. Most common of bacterial causing diarrhea consists of various pathogens such as *Vibrio cholerae*, *Shigella* sp., *Salmonella* sp.. Pathogenic *Escherichia coli* group, including enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), and enterotoxigenic *E. coli* (ETEC), is also regarded as an important causative agent [1]. Treatment of these infections normally uses antibiotics but they are expensive and can induce antibiotic-resistant pathogen strains [2]. One of alternative solutions to overcome these problems is probiotics.

Probiotics are defined as live microorganisms which when consumed in adequate amounts confer a health effect on the host [3]. Probiotics have a safety to use known as generally recognized as safe (GRAS). The most studied probiotics are lactic acid-producing bacteria, particularly *Lactobacillus* species [4, 5]. *Lactobacillus* is a microflora of human gastrointestinal tract including stomach and most abundant in intestine [6]. Recently, lactobacilli have been used as probiotics for several benefits to the host, these useful effects included antimicrobial property against various enteric pathogens causing diarrheal disease [7].

Previous studies have been reported that lactobacilli had bactericidal effects to the enteric pathogens, such as *Shigella* sp. and *Vibrio* sp.. The mechanisms of inhibition of these pathogens are involved in secreted products of lactobacilli known as reuterin [8]. Several lines of evidence also showed that *Lactobacillus* could inhibit growth of the enteric pathogens *in vitro* and *in vivo* [9-11]. In addition, the beneficial effect of probiotic lactobacilli have been shown in decreasing of frequency of infections and shortening of the duration of diarrhea in infectious disease causing by the enteric pathogens [12, 13]. Treatment of the enteric pathogens causing diarrhea in conjunction with probiotics could reduce the risk and duration of disease [14, 15]. For the efficacy of probiotics, previous report suggested that probiotics should be isolated from native population and using them in the same population [16]. In case of Thai people, it is necessary to isolate the lactobacilli from Thai population and use them as probiotics for the treatment in Thai patients. Thus, in this study, *Lactobacillus* Thai isolates were screened for the antibacterial activity against the enteric pathogens and they were also examined for synergistic activity *in vitro*.

Materials and Methods

Bacterial strains and culture conditions

The 227 *Lactobacillus* Thai isolates were kindly provided by Assoc. Prof. Dr.Somying Tumwasorn, consisting of 85 isolates from gastric biopsy and 142 isolates from throat swab [17] were cultured in de Man Rogosa Sharpe (MRS) agar (Oxoid) in anaerobic condition at 37 ^{o}C for 24-48 hours. All *Lactobacillus* Thai isolates were previously identified by 16S rRNA identification [17]. The enteric pathogens, enterohemorrhagic *E. coli* (EHEC) O157:H7 DMST 12743, enteroinvasive *E. coli* (EIEC) DMST 20971, enteropathogenic *E. coli* (EPEC) DMST 20972, enterotoxigenic *E. coli* (ETEC) DMST 20970, *Shigella flexneri* DMST 4423, *Salmonella enterica* Typhimurium ATCC 13311, *Vibrio cholerae* non-O1, non-O139 DMST 2873 and commensal/non-pathogenic *E. coli* ATCC 25922 [18, 19], representative of commensal microbiota (Department of Medical Science, Ministry of Public Health, Thailand) were cultured in Tryptone Soya agar (TSA) (Oxoid) in aerobic condition at 37 °C for 18-24 hours.

Spot-Overlay method

All *Lactobacillus* isolates were tested for antibacterial activity against the enteric pathogens and commensal/non-pathogenic *E. coli* by using spot-overlay method with modification as described previously [8]. Briefly, 24 hours-cultures of *Lactobacillus* in MRS broth in a 96-well plate were spotted (2 µL) onto the surfaces of Brain Heart Infusion (BHI) agar supplemented with 20 mM glucose in 140 mm Petri dish and incubated in anaerobic condition at 37 °C for 48 hours. The mixture of the enteric pathogens or *E. coli* ATCC 25922 containing in 7 mL of soft agar (0.75% agar) of Tryptone Soya broth (TSB) were overlaid onto each plate at a final concentration of approximately $1x10^{7}$ CFU/mL and incubated in aerobic condition at 37 °C for 24 hours. Clear zones around spots of *Lactobacillus* isolates of more than or equal to 1 mm were scored as positive. These experiments were performed in three independent determinations, each in duplicate. In order to determine the synergistic activity among *Lactobacillus* isolates, the same spot-overlay method but difference in pattern designation of spots on the plate was done. The selected strains were spotted in conjunction with other strains (the distance between each spot approximately 5 mm) randomly compared with selected strains alone.

Concentration of cultured supernatants

The *Lactobacillus* isolates were cultured in BHI broth (inoculum size = OD_{600} 0.1) in anaerobic conditions at 37 °C for 48 hours. Then the cultures were centrifuged at 5,000 rpm, 25 °C for 20 minutes (RC3C, Sorvall Instruments, Dupont). The supernatants were collected and filtrated by using 0.22 μm filter paper (Millipore). Concentrations of sterile supernatants were measured by Amicon Ultra-4 3K (Millipore) according to the manufacturer's instruction. Briefly, 4 mL supernatants were added into Amicon tube and centrifuged by swinging bucket rotor at 4000 x g, 25 °C for 30 minutes, and collected retentated recovery for next step. Ten microliters of retentated supernatants were dropped onto the enteric pathogens streaked TSA plates and incubated in aerobic condition at 37 °C for 24 hours. Clear zones on the plates were interpreted as positive and the experiments were performed in three independent determinations, each in duplicate.

Results

Antibacterial activity of *Lactobacillus* against enteric pathogens

Thirty eight isolates of *Lactobacillus* consisting of 15 isolates from gastric biopsy and 23 isolates from throat swab inhibited *S. flexneri*, and 72 isolates consisting of 22 isolates from gastric biopsy and 50 isolates from throat swab inhibited *V. cholerae*, respectively (Table 1). Thirty two isolates of *Lactobacillus* could inhibit both *S. flexneri* and *V. cholerae* (Table 1). However, all *Lactobacillus* isolates did not inhibit *S. enterica* Typhimurium, EHEC, EIEC, EPEC, ETEC and also *E. coli* ATCC 25922. These results led to the question that the inhibition zones around the spots of *Lactobacillus* occurred from antibacterial activity of single isolate alone or more than one isolate. Therefore, the *L. gasseri* B49, *L. plantarum* B64, *L. fermentum* B66, *L. plantarum* B67, *L. mucosae* B79, *L. casei* T20, *L. reuteri* T36, *L. fermentum* T37, *L. salivarius* T38, *L. salivarius* T39, *L. salivarius* T69, *L. salivarius* T70, *L. salivarius* T71, *L. mucosae* T78, and *L. mucosae* T102 were selected for further experiments to help understanding this inhibitory effect. The previous 16S rRNA identification of these lactobacilli [17] are shown in Tables 1 and 2.

Table 1 Antibacterial activity of *Lactobacillus* spp. against enteric pathogens.

Code	Species	Source [17]	Pathogen		Code	Species	Source	Pathogen	
			S. flexneri	*V. cholerae*				*S. flexneri*	*V. cholerae*
B6	*L. plantarum*	GB	+	+	T37	*L. fermentum*	TS	-	+
B7	*L. plantarum*	GB	+	+	T38	*L. salivarius*	TS	+	+
B22	*L. oris*	GB	+	+	T42	*L. salivarius*	TS	+	+
B24	*L. fermentum*	GB	-	+	T49	*L. salivarius*	TS	+	+
B36	*L. agilis*	GB	+	+	T60	*L. casei*	TS	-	+
B37	*L. salivarius*	GB	+	-	T62	*L. salivarius*	TS	-	+
B42	*L. fermentum*	GB	-	+	T69	*L. salivarius*	TS	+	+
B46	*L. fermentum*	GB	-	+	T70	*L. salivarius*	TS	+	+
B47	*L. salivarius*	GB	-	+	T71	*L. salivarius*	TS	+	+
B55	*L. salivarius*	GB	+	-	T80	*L. fermentum*	TS	-	+
B67	*L. plantarum*	GB	+	+	T89	*L. salivarius*	TS	-	+
B72	*L. fermentum*	GB	-	+	T90	*L. salivarius*	TS	-	+
B73	*L. salivarius*	GB	+	+	T96	*L. salivarius*	TS	-	+
B74	*L. salivarius*	GB	+	-	T98	*L. fermentum*	TS	-	+
B83	*L. fermentum*	GB	-	+	T99	*L. plantarum*	TS	+	+
B87	*L. plantarum*	GB	+	+	T100	*L. plantarum*	TS	+	+
B90	*L. plantarum*	GB	+	+	T103	*L. plantarum*	TS	+	+
B91	*L. salivarius*	GB	+	+	T109	*L. fermentum*	TS	-	+
B92	*L. fermentum*	GB	-	+	T111	*L. fermentum*	TS	-	+
B95	*L. gasseri*	GB	-	+	T125	*L. casei*	TS	-	+
B99	*L. fermentum*	GB	-	+	T134	*L. salivarius*	TS	-	+
B105	*L. fermentum*	GB	-	+	T141	*L. casei*	TS	+	+
B106	*L. casei*	GB	+	-	T142	*L. casei*	TS	-	+
B109	*L. salivarius*	GB	+	+	T150	*L. fermentum*	TS	-	+
B110	*L. salivarius*	GB	-	+	T152	*L. salivarius*	TS	+	+
XB7	*L. plantarum*	GB	+	+	T154	*L. salivarius*	TS	-	+
T5	*L. fermentum*	TS	-	+	T156	*L. salivarius*	TS	-	+
T6	*L. salivarius*	TS	+	+	T158	*L. salivarius*	TS	-	+
T10	*L. salivarius*	TS	+	-	T159	*L. fermentum*	TS	-	+
T14	*L. salivarius*	TS	+	+	T162	*L. salivarius*	TS	-	+
T15	*L. fermentum*	TS	-	+	T167	*L. salivarius*	TS	-	+
T16	*L. salivarius*	TS	+	+	T168	*L. salivarius*	TS	-	+
T17	*L. salivarius*	TS	+	-	T171	*L. salivarius*	TS	-	+
T18	*L. salivarius*	TS	+	+	T177	*L. fermentum*	TS	-	+
T19	*L. salivarius*	TS	+	+	T178	*L. casei*	TS	-	+
T26	*L. salivarius*	TS	+	+	T183	*L. delbrueckii*	TS	-	+
T27	*L. oris*	TS	+	+	T184	*L. salivarius*	TS	-	+
T28	*L. salivarius*	TS	+	+	T185	*L. fermentum*	TS	-	+
T32	*L. casei*	TS	+	+	XT7	*L. reuteri*	TS	+	+

GB = Human gastric biopsy

TS = Human throat swab

\+ = Clear zone ($\geq$ 1 mm)

\- = No clear zone

Table 2 *Lactobacillus* Thai isolates with synergistic activity.

	Species	Source [17]	Spot alone		Spot in conjunction		Note
			S. flexneri	*V. cholerae*	*S. flexneri*	*V. cholerae*	
B49	*L. gasseri*	GB	-	-	-	-	This group consisted of B49, B64, B66, B67, and B79.
B64	*L. plantarum*	GB	-	-	-	-	
B66	*L. fermentum*	GB	-	-	+	+	
B67	*L. plantarum*	GB	+	+	+	+	
B79	*L. mucosae*	GB	-	-	-	-	
T20	*L. casei*	TS	-	-	+	+	This group consisted of T20, T37, T38, T39, and T78.
T37	*L. fermentum*	TS	-	+	-	+	
T38	*L. salivarius*	TS	+	+	++	++	
T39	*L. salivarius*	TS	-	-	-	-	
T78	*L. mucosae*	TS	-	-	-	-	
T36	*L. reuteri*	TS	-	-	-	-	This group consisted of T36, T69, T70, T71, and T102.
T69	*L. salivarius*	TS	+	+	+	+	
T70	*L. salivarius*	TS	+	+	++	++	
T71	*L. salivarius*	TS	+	+	+	+	
T102	*L. mucosae*	TS	-	-	-	-	

GB = Human gastric biopsy

TS = Human throat swab

\+ = Clear zone ($\geq$ 1 mm)

++ = Larger clear zone than spot alone

\- = No clear zone

Synergistic activity of *Lactobacillus* against *S. flexneri* and *V. cholerae*

The results showed that the selected *Lactobacillus* isolates demonstrated the synergistic activity to inhibit *S. flexneri* and *V. cholerae*. The *L. fermentum* B66 when spotted alone did not show clear zones to *S. flexneri* and *V. cholerae*, but when spotted in conjunction with *L. gasseri* B49, *L. plantarum* B64, *L. plantarum* B67, and *L. mucosae* B79, *L. fermentum* B66 could exhibit the clear zone around itself (Fig 1, representative by *V. cholerae*). In addition, *L. salivarius* T38 and *L. salivarius* T70 when spotted in conjunction with a group of 4 isolates including *L. casei* T20, *L. fermentum* T37, *L. salivarius* T39, *L. mucosae* T78, and a group of *L. reuteri* T36, *L. salivarius* T69, *L. salivarius* T71, *L. mucosae* T102, respectively, showed much larger clear zones to *S. flexneri* and *V. cholerae* than the spot of each *Lactobacillus* alone (Fig 2, representative by *V. cholerae*). In order to determine the antibacterial substances produced by *Lactobacillus*, the bacteria were cultured in broth and the cultured supernatants were collected and concentrated. However, the concentrations of the supernatants of *L. fermentum* B66, *L. salivarius* T38, and *L. salivarius* T70 alone or co-incubated with *L. gasseri* B49, *L. plantarum* B64, *L. plantarum* B67 and *L. mucosae* B79; *L. casei* T20, *L. fermentum* T37, *L. salivarius* T39, and *L. mucosae* T78; *L. reuteri* T36, *L. salivarius* T69, *L. salivarius* T71, and *L. mucosae* T102, respectively, did not show clear zones against *S. flexneri* or *V. cholerae*.

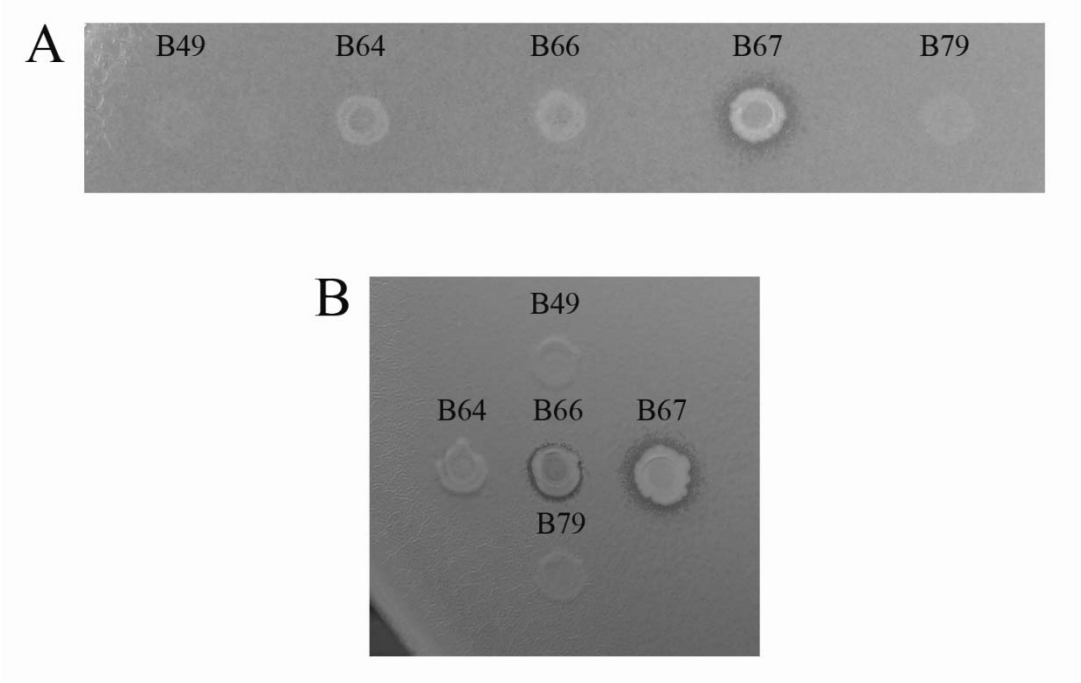

Figure 1 Synergistic activity of *Lactobacillus* against *Vibrio cholerae*. *Lactobacillus fermentum* B66 spotted alone did not show clear zone (A) while when spotted in conjunction with *L. gasseri* B49, *L. plantarum* B64, *L. plantarum* B67, and *L. mucosae* B79, *L. fermentum* B66 acquired the ability to inhibit the pathogens (B).

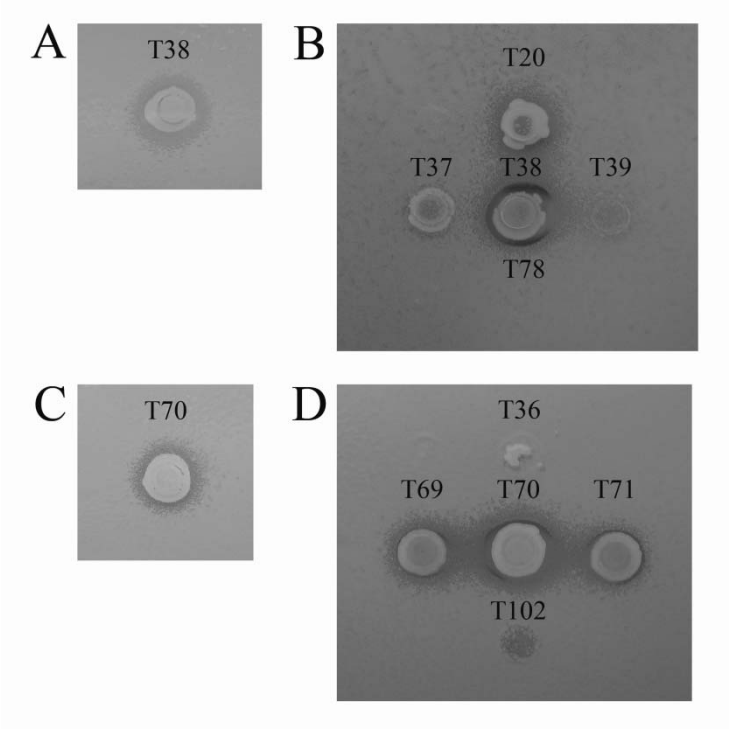

Figure 2 Enhancement of inhibition of *Vibrio cholerae* by synergistic activity of *Lactobacillus*. *Lactobacillus salivarius* T38 spotted alone (A) showed clear zone smaller than *L. salivarius* T38 spotted in conjunction with *L. casei* T20, *L. fermentum* T37, *L. salivarius* T39, and *L. mucosae* T78 (B). Alike *L. salivarius* T38, *L. salivarius* T70 spotted alone (C) showed clear zone slightly when compared with *L. salivarius* T70 spotted in conjunction with *L. reuteri* T36, *L. salivarius* T69, *L. salivarius* T71, and *L. mucosae* T102 (D).

Conclusion and Discussion

A previous study reported the protective role of treatment by using *Lactobacillus* in *S. dysenteriae* 1-induced diarrheal rat model [20]. Their results supported beneficial effect of using *Lactobacillus* as a prophylaxis agent in *Shigella*-causing disease. The previous *in vitro* study of *L. reuteri* against enteric bacterial pathogens showed that *L. reuteri* could inhibit *S. sonnei* and *V. cholerae* [8]. In the same way, the present study demonstrated that antibacterial activity of *Lactobacillus* Thai isolates could inhibit *S. flexneri* or *V. cholerae*. To examine whether these lactobacillli could possibly be used in human, the *E. coli* ATCC 25922, representative of ubiquitous microbiota [18, 19], was tested to investigate growth inhibition. All of *Lactobacillus* Thai isolates did not inhibit *E. coli* ATCC 25922, thus, they had a potential to be used as probiotics against infectious diseases caused by the enteric pathogens such as shigellosis and cholera. However, further studies are needed to confirm these beneficial effects of *Lactobacillus* whether it actually is suitable for the host.

This study demonstrated that the synergistic activity was present among the *Lactobacillus* Thai isolates. When the *Lactobacillus* isolates were spotted together to inhibit *S. flexneri* and *V. cholerae*, enhancement of inhibition against the pathogens were taken place (Fig 2). The evidence from the experiments of *L. fermentum* B66 supported this synergistic activity, *L. fermentum* B66 required *L. gasseri* B49, *L. plantarum* B64, *L. plantarum* B67 and *L. mucosae* B79 to inhibit *S. flexneri* and *V. cholerae*, while *L. fermentum* B66 alone did not inhibit the pathogens (Fig 1). In addition, *L. salivarius* T38 and *L. salivarius* T70 with the synergistic activity had greater clear zones against *S. flexneri* and *V. cholerae* when compared with *L. salivarius* T38 and *L. salivarius* T70 spotted alone (Fig 2). These results suggested that *Lactobacillus* Thai isolates with both the antibacterial and synergistic activities could inhibit *S. flexneri* and *V. cholerae* better than the antibacterial activity alone. Similarly, a previous study reported antimicrobial activity and synergistic interactions between *L. fermentum* L23 and *L. rhamnosus* L60 against urogenital pathogens, and also identified the antimicrobial compound as bacteriocin [21]. However, in the present study, the concentrated culture supernatants of these isolates could not inhibit the pathogens.

In conclusion, the synergistic activity of the *Lactobacillus* Thai isolates with antibacterial activity could enhance the inhibitory effects of *S. flexneri* and *V. cholerae* but did not kill the commensal *E. coli*. So, these beneficial properties might be used for prevention or treatment of *Shigella* or *Vibrio* causing diarrheal disease. However, further study is still needed to investigate the antibacterial substance-mediated killing of the pathogens by *Lactobacillus*, and also to understand the mechanisms that trigger *Lactobacillus* to produce the antibacterial substances to promote inhibition of the pathogens.

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