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Original Article

In Vitro Efficacy of Human-Derived Probiotic, *Lactobacillus rhamnosus* Against Pathogenic Bacteria in Fish and Frogs

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Abstract

Probiotic supplementation is now being focused as an alternative method to control fish diseases worldwide. This study investigates the *in vitro* efficacy of a human-derived probiotic, *Lactobacillus rhamnosus*. The first results of the screening for antimicrobial activity using agar spot test and disc diffusion showed that *L. rhamnosus* has a broad range against twelve isolates (n=12) of Gram-positive and Gram-negative pathogenic bacteria in fish and frogs : *Streptococcus iniae* (n=4), *Streptococcus agalactiae* (n=3), *Aeromonas hydrophila* (n=3), *Chryseobacterium indologenes* (n=1) and *Edwardsiella tarda* (n=1). Agar spot test on killed probiotic bacteria indicated that only the metabolic product of probiotic is involved in the growth inhibition of pathogenic bacteria. When confirmed by a co-culture study, the growth of all pathogenic bacteria that were cultured with a probiotic was lower than the control. All the findings suggest that *L. rhamnosus* has a high potential for inhibiting the growth of pathogenic bacteria in fish and frogs.

Keywords : fish, frog, human-derived probiotic, *Lactobacillus rhamnosus,* pathogenic bacteria

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บทคัดย่อ

ประสิทธิภาพของเชื้อโปรไบโอติก *แลคโตบาซิลลัส แลมโนซัส* ที่แยกได้จากคนต่อจุลชีพก่อโรคในปลาและกบ

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ในปัจจุบันการใช้โปรไบโอติกผสมอาหารได้รับความสนใจมากขึ้นเพื่อใช้ในการควบคุมโรคต่างๆ ในปลา ทำการศึกษาภายในห้องทดลองถึงประสิทธิภาพของโปรไบโอติก แลคโตบาซิลลัส แลมโนซัส *(Lactobacillus rhamnosus)* ที่แยกได้จากคน ทดสอบความสามารถในการยับยั้งจุลชีพในห้องปฏิบัติการด้วยวิธี agar spot และ disc diffusion พบว่า *L. rhamnosus* สามารถยับยั้งการเจริญเติบโตของเชื้อแบคทีเรียแกรมบวกและแกรมลบทั้ง 12 ไอโซเลท (isolate) ที่ก่อโรคทั้งในปลาและกบได้ คือ *สเตรปโตคอคคัส อินนิเอ้ (Streptococcus iniae)* (n=4), *สเตรปโตคอคคัส อะกาแลคติเอ้ (Streptococcus agalactiae)* (n=3), *แอโรโมนาส ไฮโดรฟิลล่า (Aeromonas hydrophila)* (n=3), *คลิสซิโอแบคทีเรียม อินโดโรจิเนส (Chryseobacterium indologenes)* (n= 1) และ *เอ็ดเวิร์ดเซลล่า ทาด้า (Edwardsiella tarda)* (n=1) และจากการทดสอบด้วยวิธี agar spot โดยใช้โปรไบโอติกแบคทีเรียที่ไม่มีชีวิต พบว่าการยับยั้งการเจริญ เติบโตของจุลชีพก่อโรค เกิดจากสารที่โปรไบโอติกแบคทีเรียผลิตขึ้นและทำการทดสอบด้วยวิธีเพาะเลี้ยงร่วม (co-culture) โดยการนำแบคทีเรียที่ก่อโรคเพาะเลี้ยงรวมกับโปรไบโอติกแบคทีเรีย พบว่าอัตราการเจริญของเชื้อแบคทีเรียก่อโรคลดลงเมื่อเทียบกับกลุ่มควบคุมจากผลการทดลองทั้งหมดพบว่าโปรไบโอติกแบคทีเรีย *L. rhamnosus* มีประสิทธิภาพสูงในการยับยั้งการเจริญเติบโตของเชื้อแบคทีเรียก่อโรคในปลาและกบ

คำสำคัญ : ปลา กบ โปรไบโอติกที่แยกได้จากคน *Lactobacillus rhamnosus* เชื้อแบคทีเรียก่อโรค

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Introduction

Among the causes of economic loss in aquaculture, infectious disease from bacteria is one of the most important. The key to success in preventing and controlling infectious diseases depends on several factors: the host immunity, the pathogen and also the environment. Two common methods of preventing and controlling are vaccination and chemotherapy, each of which can deal with only one factor. Accordingly, probiotics have become an interesting alternative way because of their direct ability to inhibit the growth of other microorganisms and modulate the host's immunity (Fuller, 1989; Gatesoupe, 1999; Verschuere et al., 2000). In the aquatic system, where hosts and pathogens share the same ecosystem, hosts are fully exposed to the pathogens (Verschuere et al., 2000). The direct effect such as inhibiting the growth of other organisms might be the main action that can occur in cultured system (Kesarcodi-Watson et al., 2008) and the ability to produce inhibitory compounds of live probiotic bacteria is one of the important actions that results in the growth inhibition of other microorganisms (Balcazar et al., 2006; Kesarcodi-Watson et al., 2008). Among probiotic candidates, Lactobacilli have the longest history of use (Fuller,. 1989). Various health effects for human have been attributed to *Lactobacillus rhamnosus*, such as the prevention of acute diarrhoea in children, the prevention of antibiotic-associated diarrhoea and the prevention and treatment of allergies, lowering cholesterol levels and the immune stimulation (Majamaa and Isolauri, 1997; Anuradha et al., 2005). In recent years, there has been great interest in the use of lactic acid bacteria (LAB) and their metabolic products as potential probiotics in aquaculture since LAB probiotics are considered safe for fish as a human food and have the ability to fight against harmful pathogens directly and indirectly (Gatesoupe, 2008). The objective of the present

study is to determine the *in vitro* efficacy of *Lactobacillus rhamnosus*, a human-derived probiotic that has been used in humans to control gastrointestinal diseases and some bacterial-infectious diseases of fish and frogs.

Materials and Methods

Bacterial strain and culture medium

L. rhamnosus (ATCC 53103) was cultured at 37°C on De Man, Rogosa and Sharpe (MRS) agar with 0.3% $CaCO_3$ and modified-MRS medium (M-MRS medium), a suitable medium for lactic acid bacteria. Twelve isolates of pathogenic bacteria (n=12) were cultured at 30°C on trypticase soy agar (TSA) and on trypticase soy borth (TSB). All pathogenic bacteria were isolated from organs of diseased fishes and frogs in Thailand and Japan (Table 1). All isolated bacteria were identified by the conventional biochemical method and confirmed by polymerase chain reaction (PCR).

Agar spot test with live probiotic bacteria

L. rhamnosus from an overnight culture in MRS broth was spotted on the surface of Modified-MRS (M-MRS) agar (possessing 2% dextrose for decrease producing organic acid) and TSA, subsequently incubated at 37°C for 24 h to allow the development of colonies. After 24 h of culture, 50 µl of each of the pathogenic bacteria was inoculated in semi-solid TSA (TSB with yeast extract of 0.6% + agar 0.75%) and was poured over the M-MRS agars and TSA. The plates were incubated at 30°C for 24 h and checked for an inhibition zone. The inhibition zones were classified as (-) for no visible inhibition, (+) for 0.5 to 6 mm inhibition, (++) for 7 to 12 mm, and (+++) for more than 12 mm inhibition. (Perea Velez et al., 2007)

Agar spot test with dead probiotic bacteria

L. rhamnosus from an overnight culture in MRS broth was centrifuged at 5,000 g for 15 min. to remove the MRS broth. The *L. rhamnosus* cells were killed by 10% formalin for 30 min and washed with phosphate buffer saline (PBS) 3 times. The dead cells were spotted on the surface of M-MRS agar and overlaid with each pathogenic bacterium following the same method as above. The plates were incubated at 30°C for 24 h and checked for their inhibition zone.

Disc diffusion assay

Free cell supernatant was prepared from a 72 h culture of *L. rhamnosus* in M-MRS and MRS broth. Cells were removed by centrifuging at 5000 g for 15 min in

Table 1 Fish and frogs pathogens used in this study.

Isolation No.	Pathogens	Host/organs	Location
1. *S. iniae* I	*S. iniae*	fish/kidney	Japan
2. *S. iniae* II	*S. iniae*	fish/liver	Japan
3. *S. iniae* III	*S. iniae*	fish/brain	Thailand
4. *S. iniae* IV	*S. iniae*	fish/kidney	Thailand
5. *S. agalactiae* I	*S. agalactiae*	fish/kidney	Thailand
6. *S. agalactiae* II	*S. agalactiae*	fish/liver	Thailand
7. *S. agalactiae* III	*S. agalactiae*	fish/spleen	Thailand
8. *A. hydrophila* I	*A. hydrophila*	mixed breed frog/ blood	Thailand
9. *A. hydrophila* II	*A. hydrophila*	mixed breed frog/ liver	Thailand
10. *A. hydrophila* III	*A. hydrophila*	fish/spleen	Thailand
11. *C. indologenes* I	*C. indologenes*	mixed breed frog/ eyeball	Thailand
12. *E. tarda* I	*E. tarda*	fish/liver	Japan

sterile condition, the supernatant fluid was filtered through a filter with 0.22 μm pore size.

Five sterile blank paper discs were placed on the Muller Hilton agar which was inoculated with each pathogenic bacterium. Then, 100 μl of the filtered supernatants of *L. rhamnosus* were applied on the paper discs (Figure 1). Plates were incubated at 30°C for 24 h and observed for their inhibition zone.

Growth inhibition by co-culture assay

Pathogenic bacteria (*S. iniae* II, *S. agalactiae* I, *C. indologenes* I, *E. tarda* I, *A. hydrophila* II) were grown to lag phase in their suitable media. One hundred (100) μl ($1x10^7$ CFU/ml) of each cultured pathogenic bacteria were inoculated in 10 ml of TSB with and without 100 μl ($1x10^7$ CFU/ml) of cultured *L. rhamnosus*. After 24 h incubation at 37°C, the colony forming unit of each pathogenic bacterium and *L. rhamnosus* was counted by using MRS agar for *L. rhamnosus* and TSA for pathogenic bacteria. The results were expressed in percentage for each pathogen growth with *L. rhamnosus* by co-culture method compared with each pathogen growth without *L. rhamnosus* (control).

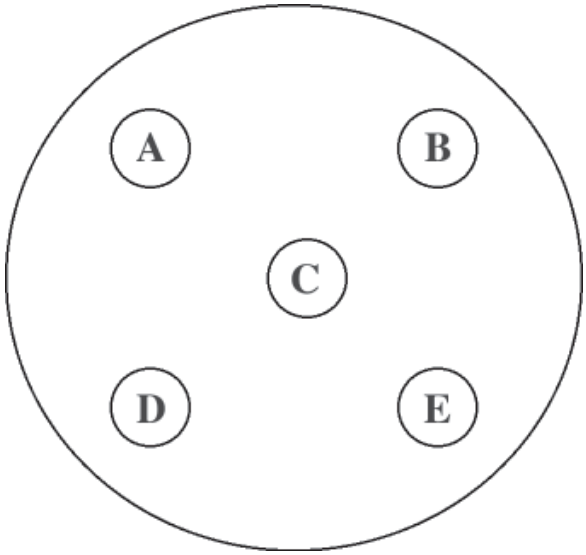

Figure 1 Disc diffusion assay: A: supernatant from M-MRS broth, B: M-MRS broth, C: supernatant from M-MRS broth adjusted pH to 6.5, D: MRS broth, E: supernatant from MRS broth

Results

Agar spot test (Table 2): By using agar spot test on M-MRS agar and TSA, *L. rhamnosus* inhibited the growth of all fish pathogens by producing a radius of at least 0.5 ml. For agar spot test on M-MRS agar the highest susceptible species was *A. hydrophila* I. The

Table 2 Inhibition zone of agar spot test on M-MRS and TSA and inhibition zone of disc diffusion assay.

	Agar spot test		Disc diffusion assay
	M-MRS	TSA	
	inhibition zone	inhibition zone	inhibition zone
S. iniae I	+	+	+
S. iniae II	+	++	++
S. iniae III	+	+	+
S. iniae IV	++	+	+
S. agalactiae I	++	+	+
S. agalactiae II	+	+	+
S. agalactiae III	+	+	+
A. hydrophila I	+++	+	+++
A. hydrophila II	++	++	+
A. hydrophila III	++	+	+
C. indologenes I	++	+	+
E. tarda I	++	+	+

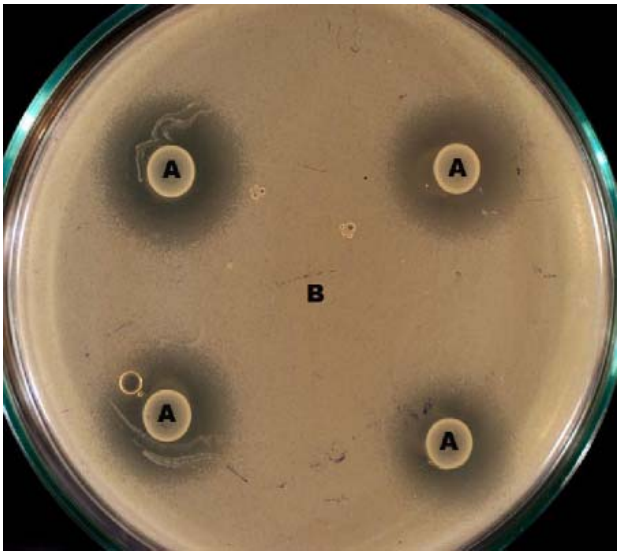

Figure 2 Agar spot test on M-MRS agar
A: *L. rhamnosus*,
B: *S. iniae* IV

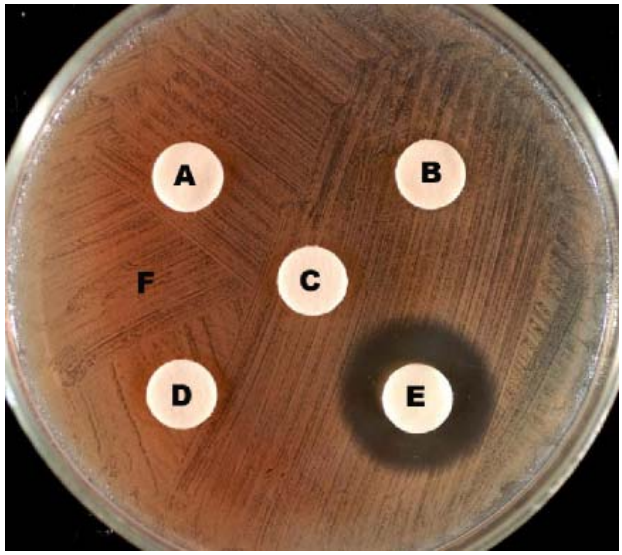

Figure 3 Disc diffusion assay
A: supernatant from M-MRS broth, B:M-MRS broth,
C: supernatant from M-MRS broth adjusted pH to 6.5,
D: MRS broth, E: supernatant from MRS broth
F: *A. hydrophila* I

highest susceptible species from the agar spot test on TSA was *S. iniae* II. There was no inhibition zone of any bacteria from the agar spot test with killed probiotic bacteria.

Disc diffusion assay: Free cell supernatant from a 72 h culture of *L. rhamnosus* in MRS broth inhibited growth of all fish and frog pathogens by producing a radius of at least 0.5 ml. All the results of disc diffusion assay are shown in Table 2. The highest susceptible species are *S. iniae* II and *A. hydrophila* I. When using supernatant from a 72 h culture of *L. rhamnosus* in M-MRS broth with adjusted pH to 6.5 and untouched supernatant from a 72 h culture of *L. rhamnosus* in M-MRS broth, there was no inhibition zone from either of them (Figure 3).

Co-culture assay: After having incubated each pathogen with probiotic bacterium for 24 h, the growth of each pathogen was lower than the control (Table 3). The lowest to the highest percent growth were as follows *S. iniae* II, *E. tarda* I, *C. indologenes* I, *A. hydrophila* II and *S. agalactiae* I

Table 3 Effect of probiotic on the growth of pathogenic bacteria.

Pathogenic bacteria	% Growth in co-culture
S. iniae II	28.70 %
S. agalactiae I	86.50 %
A. hydrophila II	83.78 %
C. indologenes I	67.29 %
E. tarda I	55.56 %

Discussion

The present study on the agar spot test of *L. rhamnosus* shows the efficacy of antimicrobial activity over all the aquatic pathogenic bacterial species used in this study. The agar spot test using killed probiotic bacteria, which showed no inhibition zones, clearly suggests that only the metabolic products, not the cells of probiotic bacteria, are involved in the growth inhibition of pathogenic bacteria. Lactic acid bacteria are known for their ability to produce inhibitory substrates such as hydrogen peroxide, organic acid and bacteriocin-like products, which are antimicrobials (De Vuyst and Leroy, 2007). The result of disc diffusion assay which showed

the inhibition zone only when the disc was applied with the supernatant from MRS broth, may suggest that the main antimicrobial substance is the organic acid because when using supernatant from M-MRS broth (which decease producing of organic acid) there was no inhibition zone. In addition, the result from the agar spot test on TSA, which is not a suitable medium for lactic acid bacteria, might indicate that even in an unfavorable environment *L. rhamnosus* still can produce antimicrobial substances. The potential for reducing the growth of pathogenic bacteria was confirmed by co-culture study. Here the probiotic and each pathogenic bacterium were cultured together in TSB. In all co-culture tubes the growth of pathogenic bacterium was lower when compared with the control tube in which just pathogenic bacteria were cultured. This is related to a previously study (Nikoskelainen et al., 2001). The mechanisms involved, are not only the antimicrobial substance that is produced from *L. rhamnosus* and the change of pH in the co-culture tube, but also the ability of *L. rhamnosus* in competition for nutrients (Verschuere et al., 2000).

From our study *L. rhamnosus*, a human-derived probiotic bacterium, is a promising probiotic candidate to be used in aquaculture with regard to its high potential against aquatic pathogenic bacteria *in vitro*. However, probiotic in aquaculture have many different properties from probiotics in humans. Information about the ability to adhere to host cells, their competitive exclusion stability in fish intestines, and the interaction between host-microbes *in vivo* is further needed.

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