รายงานวิจัยฉบับสมบูรณ์

โครงการ กลไกการป้องกันและการสร้างภูมิคุ้มกันของอาหารเสริมโปรไบโอติกที่แยกได้จากมนุษย์ต่อโรคติดเชื้อแบคทีเรียในปลานิล

โดย

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และคณะ

2 กรกฎาคม 2552

Abstract

Project Code: MRG5080265

Project Title: Protective mechanism and immunity of human-derived probiotic-supplemented diet against bacterial disease in tilapia (*Oreochromis niloticus*)

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Probiotic supplementation is now being focused as an alternative method to control fish diseases. This study investigated the efficacy of the probiotic in growth performance and the protective effects of *Lactobacillus rhamnosus*-supplemented diet against *Streptococcus iniae* and *Streptococcus agalactiae* after intraperitoneal challenging at a concentration of $2x10^8$ CFU/fish. After feeding for 14 and 30 days, the probiotic group had better weight gain, specific growth rate and feed utilization (FCR). In addition, the villus height in probiotic group was greater than control group. An *in vitro* study on antimicrobial activity using agar spot test and disc diffusion showed that *L. rhamnosus* strongly inhibited the growth of *S. iniae* (four strains) and *S. agalactiae* (two strains). In the *in vivo* study, the relative percent survival (RPS) in the probiotic group challenged with *S. iniae* was 62.5 and RPS in the probiotic group challenged with *S. agalactiae* was 46.43. Histopathology, both of probiotic groups had more evidence in number of melano-macrophage center in head kidney at 3 and 7 DPI. *L. rhamnosus* induced significantly higher IL beta and TNF alpha expression in head kidney and spleen, implying probiotic involvement through these mediators of immune response. The results of this study suggested that supplementation of *L. rhamnosus* in feed could enhance immunity against streptococcosis and enhance the growth performance in tilapias.

Key words: Probiotic, *Lactobacillus rhamnosus*, growth performance, antimicrobial activity, immunity

บทคัดย่อ

รหัสโครงการ: MRG5080265

ชื่อโครงการ: กลไกการป้องกันและการสร้างภูมิคุ้มกันของอาหารเสริมโปรไบโอติกที่แยกได้จากมนุษย์ต่อโรคติดเชื้อแบคทีเรียในปลานิล

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ในปัจจุบันการใช้โปรไบโอติกผสมอาหารได้รับความสนใจมากขึ้นเพื่อใช้ในการควบคุมโรคต่างๆในปลา การศึกษานี้ได้ศึกษาประสิทธิภาพของโปรไบโอติก (*Lactobacillus rhamnosus*) ต่อการเจริญเติบโตของปลานิลและการควบคุมโรคที่เกิดจากเชื้อ *Streptococcus iniae* และเชื้อ *Streptococcus agalactiae* โดยใช้เชื้อปริมาณ $2x10^8$ เซลล์ต่อมิลลิลิตรฉีดเข้าช่องท้อง ผลการทดลองการให้อาหารเสริมโปรไบโอติก เป็นระยะเวลา 14 และ 30 วัน พบว่ากลุ่มโปรไบโอติกมีการเพิ่มขึ้นของน้ำหนักตัว อัตราการเจริญเติบโต และอัตราการแลกเนื้อดีกว่ากลุ่มควบคุม นอกจากนี้ในกลุ่มโปรไบโอติกยังมีความยาวของวิลไลมากกว่ากลุ่มควบคุมอีกด้วย ในการทดสอบความสามารถในการยับยั้งจุลชีพในห้องปฏิบัติการด้วยวิธี agar spot พบว่า *L. rhamnosus* สามารถยับยั้งการเจริญเติบโตของเชื้อ *S. iniae* (4 สายพันธุ์) และเชื้อ *S. agalactiae* (2 สายพันธุ์) เมื่อทำการฉีดเชื้อพิษ (เชื้อ *S. iniae* และเชื้อ *S. agalactiae*) เข้าปลานิล พบว่ากลุ่มโปรไบโอติกมีเปอร์เซ็นต์การรอดชีวิตสัมพัทธ์ (RPS) เท่ากับ 62.5 และ 46.43 ตามลำดับ ในทางจุลพยาธิวิทยาพบว่ากลุ่มโปรไบโอติกมีจำนวน melano-macrophage center มากขึ้นบริเวณไตส่วนหน้าในวันที่ 3 และ 7 หลังการฉีดเชื้อ จากผลการทดลอง *L. rhamnosus* สามารถกระตุ้นการแสดงออกของจีนที่ตอบสนองทางภูมิคุ้มกัน Interlukin 1 และ Tumor necrotic factor ได้อย่างมีนัยสำคัญ การเสริมโปรไบโอติก (*L. rhamnosus*) ในอาหารสามารถเพิ่มการตอบสนองของระบบภูมิคุ้มกันต่อโรคสเตรปโตคอคโคซิสและเพิ่มการเจริญเติบโตได้ในปลานิล

คำสำคัญ: โปรไบโอติก *Lactobacillus rhamnosus* การเจริญเติบโต ความสามารถในการยับยั้งจุลชีพ ระบบภูมิคุ้มกัน

หน้าสรุปโครงการ (Executive Summary)
ทุนพัฒนาศักยภาพในการทำงานของอาจารย์รุ่นใหม่

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1. **ชื่อโครงการ (ภาษาไทย)** กลไกการป้องกันและการสร้างภูมิคุ้มกันของอาหารเสริมโปรไบโอติกที่แยกได้จากมนุษย์ต่อโรคติดเชื้อแบคทีเรียในปลานิล

(ภาษาอังกฤษ) Protective mechanism and immunity of human-derived probiotic-supplemented diet against bacterial disease in tilapia (*Oreochromis niloticus*)

2. ชื่อ หัวหน้าโครงการวิจัย หน่วยงานที่สังกัด ที่อยู่ หมายเลขโทรศัพท์ โทรสาร และ e-mail

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3. สาขาวิชาที่ทำการวิจัย พยาธิวิทยาภูมิคุ้มกันในสัตว์น้ำ

5. ระยะเวลาดำเนินการ 2 ปี

6. ปัญหาที่ทำการวิจัย และความสำคัญของปัญหา

การบริโภคอาหารปลอดสารพิษและสารเคมีตกค้างเป็นสิ่งสำคัญที่ทางรัฐบาลตระหนักและให้ความสำคัญอย่างยิ่ง โดยเฉพาะแหล่งอาหารโปรตีนที่ได้จากสัตว์น้ำซึ่งกำลังได้รับความนิยมอย่างแพร่หลายในปัจจุบันและอนาคต แต่การเลี้ยงสัตว์น้ำในประเทศไทยเพื่อบริโภคหรือผลิตเพื่อส่งออกในปัจจุบันมักมีการใช้ยาปฏิชีวนะหรือสารเคมีที่อันตรายต่อผู้บริโภคเข้ามาใช้ในวงจรการเลี้ยง ทำให้เกิดการตกค้างของยาปฏิชีวนะหรือสารเคมีภายในเนื้อสัตว์ส่งผ่านไปยังมนุษย์ ส่งผลทำให้จุลินทรีย์เกิดการดื้อยา เพิ่มความลำบากในการควบคุมโรคต่อตัวสัตว์และมนุษย์ทั้งทางตรงและทางอ้อม ดังนั้นแนวความคิดการใช้จุลินทรีย์ที่มีประโยชน์เพื่อควบคุมจุลินทรีย์ที่ก่อโรค เช่น การใช้จุลินทรีย์โปรไบโอติกผสมในอาหารสัตว์น้ำจึงเป็นทางเลือกหนึ่งของการพัฒนาเกษตรอินทรีย์เพื่อเพิ่มผลผลิตสัตว์น้ำให้ปลอดภัยจากอันตรายและสารเคมีตกค้าง ส่งผลให้เกิดความปลอดภัยกับผู้บริโภคโดยตรง

การใช้จุลินทรีย์โปรไบโอติกในสัตว์น้ำในปัจจุบันมีการศึกษากันอย่างแพร่หลายและเป็นที่ยอมรับในเชิงวิชาการถึงความสามารถในการเพิ่มผลผลิต การป้องกันโรคและการเสริมสร้างภูมิคุ้มกันต่อตัวสัตว์ ลดการใช้และนำเข้ายาปฏิชีวนะ และลดการตกค้างของยาปฏิชีวนะภายในเนื้อสัตว์ แต่จุลินทรีย์ส่วนมากที่นำมาใช้เป็นผลิตภัณฑ์โปรไบโอติกในสัตว์น้ำนั้นมีมากมายแตกต่างกันตามชนิดและสายพันธุ์ และส่วนมากเป็นจุลินทรีย์สายพันธุ์ที่แยกได้จากสัตว์น้ำเองโดยตรง ซึ่งจุลินทรีย์สายพันธุ์เหล่านี้ส่วนมากยังไม่มีการทดสอบถึงความปลอดภัยต่อมนุษย์โดยตรง และการผสมจุลินทรีย์โปรไบโอติกในอาหารนั้นต้องใช้ในปริมาณมากเพื่อให้เกิดประสิทธิผลโดยสมบูรณ์ (Gatesoupe, 1999) จึงต้องระมัดระวังเรื่องการปนเปื้อนและการตกค้างของจุลินทรีย์เหล่านี้ในอาหารหรือผลิตภัณฑ์ที่ได้จากสัตว์น้ำ โดยเฉพาะการบริโภคอาหารหรือผลิตภัณฑ์สัตว์น้ำที่สด ยังไม่มีการแปรรูป ดังนั้นเพื่อความปลอดภัยต่อผู้บริโภคโดยตรง การใช้จุลินทรีย์ที่ได้จากมนุษย์โดยตรงมาผสมในอาหารสัตว์น้ำจึงเป็นทางเลือกหนึ่งของการพัฒนาเกษตรอินทรีย์ที่ใช้เพื่อการผลิตสัตว์น้ำและเกิดความปลอดภัยกับผู้บริโภค นอกจากนี้การใช้จุลินทรีย์โปรไบโอติกที่เป็นประโยชน์จากมนุษย์ยังส่งผลดีโดยตรงต่อผู้บริโภค ดังที่รายงานไว้ในเอกสารวิชาการมากมายถึงประโยชน์ของจุลินทรีย์โปรไบโอติกในอาหารและเครื่องดื่ม เช่น จุลินทรีย์แลคโตบาซิลลัส ช่วยปรับสมดุลย์ในทางเดินอาหาร ลดอาการท้องเสีย (Isolauri et al., 1993; Pant et al., 1996) ลดการอักเสบ ลดความรุนแรงของอาการแพ้ต่างๆ (Kalliomaki et al., 2003) และช่วยลดอุบัติการณ์ปัญหาโรคเหงือกอักเสบและฝันผุ (Nase et al., 2001) เป็นต้น

การศึกษาถึงประสิทธิผลของจุลินทรีย์โปรไบโอติกที่แยกได้จากมนุษย์ต่อการเพิ่มผลผลิต การป้องกันโรคและสร้างเสริมภูมิคุ้มกันต่อคุณภาพชีวิตของสัตว์น้ำ ในปัจจุบันกำลังได้รับความนิยม เช่น รายงานการใช้ประโยชน์จากโปรไบโอติกแบคทีเรียที่แยกได้จากมนุษย์เพื่อป้องกันโรคติดเชื้อแบคทีเรียที่สำคัญในปลา เช่น โรคติดเชื้อ Furunculosis (Nikoskelainen et al., 2001) โรคติดเชื้อ Vibriosis (Gildberg and Mikkelsen, 1998) และโรคติดเชื้อ Edwardsiellosis (Pirarat et al., 2006) อย่างไรก็ตามเนื่องจากกลไกการทำงานของจุลินทรีย์โปรไบโอติกต่อการควบคุมจุลินทรีย์ก่อโรคมีรายงานไว้มากมาย ส่วนมากเป็นการศึกษาผลโดยตรงของเชื้อจุลินทรีย์โปรไบโอติกที่มีต่อเชื้อจุลินทรีย์ก่อโรค เช่น การแย่งจับยึดเกาะบริเวณเยื่อบุผิวลำไส้ การผลิตสารเคมีบางอย่างโดยตรงเพื่อทำลายจุลินทรีย์ก่อโรค หรือการแย่งอาหาร น้ำหรือแร่ธาตุต่างๆภายในทางเดินอาหาร ปรับเปลี่ยนสภาพแวดล้อมในน้ำไม่เหมาะแก่การก่อโรคของจุลินทรีย์ (Verschuere et al., 2000) แต่กลไกสำคัญที่จุลินทรีย์โปรไบโอติกกระตุ้นให้ร่างกายสัตว์น้ำเองสร้างภูมิคุ้มกันต่อเชื้อจุลินทรีย์ก่อโรคแต่ละชนิดนั้นยังมีการศึกษาไม่เด่นชัด เนื่องจากจุลินทรีย์ก่อโรคในสัตว์น้ำมีหลายชนิด กลไกการก่อโรคและพยาธิกำเนิดของจุลินทรีย์แต่ละชนิดนั้นแตกต่างกันอย่างสิ้นเชิง การศึกษาวิจัยในครั้งนี้มุ่งประเด็นที่ความสำคัญของโปรไบโอติกแบคทีเรียที่แยกได้จากมนุษย์ต่อการกระตุ้นให้ร่างกายสร้างภูมิคุ้มกัน การป้องกันการติดเชื้อจุลินทรีย์ก่อโรคในสัตว์น้ำ และศึกษากลไกเชิงลึกที่มีผลต่อระบบป้องกันภัยในตัวสัตว์น้ำ

8. วัตถุประสงค์

1.เพื่อศึกษาประสิทธิภาพของอาหารเสริมโปรไบโอติกแบคทีเรียที่แยกได้ในมนุษย์ต่อการควบคุมโรคติดเชื้อแบคทีเรียในปลานิล

2.ทราบกลไกการป้องกันโรคและการกระตุ้นระบบภูมิคุ้มกันของอาหารเสริมโปรไบโอติกต่อเชื้อแบคทีเรียก่อโรคในปลานิล

9. ระเบียบวิธีวิจัย

9.1 **สัตว์ทดลอง** ปลานิล (*Oreochromis niloticus*) สายพันธุ์จิตรลดา 3 น้ำหนักเฉลี่ย 100 กรัม

จุลินทรีย์โปรไบโอติก : *Lactobacillus rhamnosus* GG

จุลินทรีย์ก่อโรค : *Streptococcus iniae*, *Streptococcus agalactiae*

วิธีการทดลอง

แบ่งกลุ่มปลาทดลองเป็น 4 กลุ่ม ๆละ 30-50 ตัว ดังนี้

กลุ่มที่ 1 กลุ่มปลาควบคุมปกติ

กลุ่มที่ 2 กลุ่มปลาที่ได้รับจุลินทรีย์โปรไบโอติกในอาหาร

กลุ่มที่ 3 กลุ่มปลาที่ได้รับเชื้อพิษ

กลุ่มที่ 4 กลุ่มปลาที่ได้รับจุลินทรีย์โปรไบโอติกในอาหารและได้รับเชื้อพิษ

ศึกษาประสิทธิภาพของจุลินทรย์โปรไบโอติก โดยการผสมลงในอาหารปลา วันละ 2 ครั้งใช้เวลา 30 วัน หลังจากนั้นทดลองให้เชื้อจุลินทรีย์ก่อโรคแล้วประเมินต่ออีก 14 วัน ทำการเก็บตัวอย่างปลาทุกๆ 3 วัน ครั้งละ 3 ตัว/กลุ่ม หลังจากได้รับเชื้อจุลินทรีย์ก่อโรค ทำการทดลองซ้ำอีก 1 ครั้ง โดยใช้ตัวอย่างเท่าเดิม นำตัวอย่างปลาที่เก็บในแต่ละครั้งมาศึกษาทางห้องปฏิบัติการ ประกอบด้วย

การศึกษาทางมหพยาธิวิทยา

1. ทำการวางยาสลบปลาโดยใช้ สารสกัดสมุนไพร (clove oil) และการุณฆาต ในวันสิ้นสุดการทดลอง
2. ทำการเจาะเก็บเลือดจาก caudal vein ใส่สารป้องกันการแข็งตัวชนิด Heparin ปั่นแยกซีรัม และเก็บรักษาสภาพ ในตู้แช่ -20 ซ.
3. ทำการชันสูตรตัวอย่างปลาในแต่ละกลุ่ม เพื่อศึกษารอยโรคทางมหพยาธิวิทยา ถ่ายภาพและบันทึกรอยโรคที่สำคัญ
4. ทำการเก็บตัวอย่างอวัยวะ ได้แก่ สมอง เหงือก ตับ ไต และ ทางเดินอาหาร โดยแบ่งเป็น2 ส่วนคือ

ส่วนที่ 1 เก็บรักษาสภาพในน้ำยา 4% glutaraldehyde เพื่อศึกษาทางจุลพยาธิวิทยา

ส่วนที่ 2 เก็บรักษาสภาพ ในตู้แช่เยือกแข็ง -80 ซ. เพื่อศึกษาการแสดงออกของจีนที่ตอบสนองทางภูมิคุ้มกัน เช่น Interlukin 1 (IL-1), Tumor necrotic factor (TNF) เป็นต้น

การเตรียมอาหารผสมจุลินทรีย์โปรไบโอติก

นำเชื้อจุลินทรีย์โปรไบโอติกที่แยกได้จากมนุษย์มาเพาะบนอาหารเลี้ยงเชื้อ และบ่มที่อุณหภูมิ 30-35 องศาเซลเซียสนาน 48 ชั่วโมง ทำการปั่นล้างด้วย phosphate buffer saline ปลอดเชื้ออย่างน้อย 3 ครั้ง นำมาสเปรย์ผสมในอาหารสัตว์น้ำและเก็บไว้ที่อุณหภูมิ -4 องศาเซลเซียส อาหารที่ผสมจุลินทรีย์โปรไบโอติกแล้วจะใช้ให้หมดภายใน 2 วัน

การศึกษาการเปลี่ยนแปลงทางจุลพยาธิวิทยา

ตัวอย่างอวัยวะได้แก่ สมอง เหงือก ตับ ไต และทางเดินอาหาร ที่เก็บรักษาสภาพในน้ำยา 4% glutaraldehyde นำมาผ่านขั้นตอนการเตรียมตัวอย่างทางฮิสโตเทคนิค ฝังในพาราฟิน และตัดให้มีความหนาประมาณ 4-6 um ย้อมสี Hematoxylin & Eosin เพื่อศึกษารอยโรคทางจุลพยาธิวิทยา

การตรวจการแสดงออกของจีนที่มีผลต่อระบบภูมิคุ้มกัน (Immune related genes)

การตรวจการแสดงออกของ mRNA ของยีนที่มีผลต่อการหลั่ง cytokine กระตุ้นการอักเสบ โดยวิธี Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) โดยใช้ตัวอย่างเนื้อเยื่อที่เก็บรักษาที่ -80 ซ. ทำการสกัด RNA โดยใช้ ชุดสกัดสำเร็จรูป Trizol RNA extraction Kit จากนั้นนำเข้าสู่ปฏิกิริยา Reverse Transcriptase reaction ให้เป็น cDNA โดยใช้ cDNA extraction kit ทำปฏิกิริยาลูกโซ่โพลิเมอเรส เพิ่มจำนวนชิ้นส่วน DNA ด้วยเครื่อง thermocycler หลังจากนั้นทำการตรวจสอบขนาดชิ้นส่วน DNA ด้วยวิธีอิเลคโตโฟเรซีส บน agarose gel ชิ้นส่วนของ DNA บนเจล สามารถมองเห็นด้วยการย้อมด้วย ethidium bromide

รายละเอียดโครงการวิจัย

Importance and rational

“A live microbial feed supplement which beneficially affects the host animals by improving its intestinal microbial balance” (Fuller, 1989) is a common term of probiotic in the past which sometimes added “mono- or mixed culture of live microorganisms”(Havenaar and Huis in't Veld, 1992) and “microbial cell preparations or components of microbial cells” (Salminen *et al*., 1999). For aquaculture, Gatesoupe (1999) redefined the word probiotic as “Microbial cells that are administered in such a way as to enter the gastrointestinal tract and to be kept alive, with the aim of improving” and Verschuere *et al*., 2000, gave additional definitions including “The ability of a probiotic to modify the “Host-associated or ambient microbial community” and “To improve the quality of its surroundings, both of which can be considered as biocontrol.” In aquaculture, there are many studies mentioning the efficacy of probiotics as a growth promoter, for example, some treatments with lactic acid bacteria increased the production of rotifers and the growth of turbots and Japanese flounders (Gatesoupe, 1989, 1991); *Bacillus subtilis* and *Lactobacillus acidophilus* as a dietary supplement gave a greater growth performance in tilapias (Aly *et al*., 2008). The principle actions of probiotics that brings out better growth performance are reducing stress, protecting a host from intestinal disorder by preventing adhesion from pathogen, inhibiting pathogenic microorganisms, enhancing the host immune response (Fuller, 1989; Gatesoupe, 1999; Verschuere *et al*., 2000) and improving nutrition (Balcazar *et al*., 2006; Fuller, 1989; Planas *et al*., 2004; Suzer *et al*., 2008).

The key to success of preventing and controlling infectious diseases depends on several factors; the host immunity, the pathogen and also the environment. In the aquatic system, which the hosts and the pathogens share the same ecosystem, the hosts are fully exposed to the pathogens (Verschuere *et al*., 2000). The use of probiotic bacteria has become an interesting alternative way because of their ability of inhibiting growth of other microorganisms and modulating the host immunity (Fuller, 1989; Gatesoupe, 1999; Verschuere *et al*., 2000). The direct effect such as inhibiting growth of other organisms might be the main action that could occur in cultured system (Kesarcodi-Watson *et al*., 2008). 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). Since Lactic acid bacteria (LAB) probiotics are considered safe for food fish and have ability to fight against harmful pathogens directly and indirectly, they are now being used as an alternative method to control diseases. *Lactobacillus rhamnosus* (Lactic acid bacteria) is a human-derived probiotic that has been used in humans to control gastrointestinal disease and some bacterial-infection diseases.

This study was conducted to assess the efficacy of a human-derived probiotic, *L. rhamnosus*, on growth performance and the ability to fight against aquatic pathogenic bacteria, *S. iniae* and *S. agalatiae* in tilapia.

Materials and methods

***In Vitro* study**

Probiotic and pathogenic bacteria

L. rhamnosus (ATCC 53103) was cultured at 37º C on De Man, Rogosa and Sharpe (MRS)(Difco™, France)(a suitable media for lactic acid bacteria) De Man *et al.,*1960) agar with 0.3% $CaCO_3$. Four strains of *S. iniae* and two strains of *S. agalactiae* from laboratory collection confirmed by PCR were cultured at 30 º C on Tryptic soy agar (TSA) a suitable media for pathogen bacteria.

***In vitro* antimicrobial activity**

An agar spot test was used to screen for antibacterial properties of *L. rhamnosus* against pathogenic bacteria. In a separate experiment, *L. rhamnosus* was spotted on the surface of Modified-MRS agar (2g dextrose for decrease producing lactic acid) and TSA.

An agar well diffusion assay and an agar spot test with killed probiotic bacteria were used to find out what element/s was/were the cause/causes of the inhibition.

Agar spot test

L. rhamnosus from an overnight culture (24h.) in MRS broth was spotted on the surface of M-MRS agar and TSA incubated at 37°C for 24 hours to allow the development of the colonies. After 24 h., 50 µl of each strain of *S. iniae* and *S. agalactiae* (5×10^6cfu/ml) were 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 (spotted with grown *L. rhamnosus*). The plates were incubated at 30°C for 24 h. and checked for inhibition zone. (n=8, Data were expressed as a mean ± S.D)

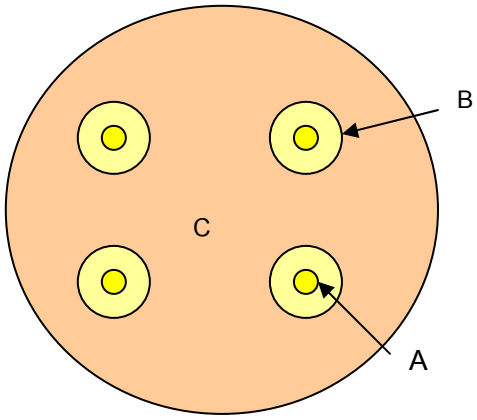


Fig 1 Agar spot test: A=*L. rhamnosus*, B=inhibition zone, C= pathogenic bacterium

Agar spot test with killed probiotic bacteria

L. rhamnosus from an overnight culture in MRS broth was centrifuged at 5,000g 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 cells were spotted on the surface of M-MRS agar and overlaid with each strain of *S. iniae* and *S. agalactiae* in the same method as above. The plates were incubated at 30°C for 24 h. and checked for inhibition zone. (n=8, Data were expressed as a mean ± S.D)

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

Five sterile blank paper discs were placed on the Muller Hilton agar which was inoculated with strains of *S. iniae* and *S. agalactiae.* Then, 100 μl of the filtered supernatant of *L. rhamnosus* were applied on the paper discs. Plates were incubated at 30°C for 24 h. and observed for inhibition zone. (n=8, Data were expressed as a mean ± S.D)

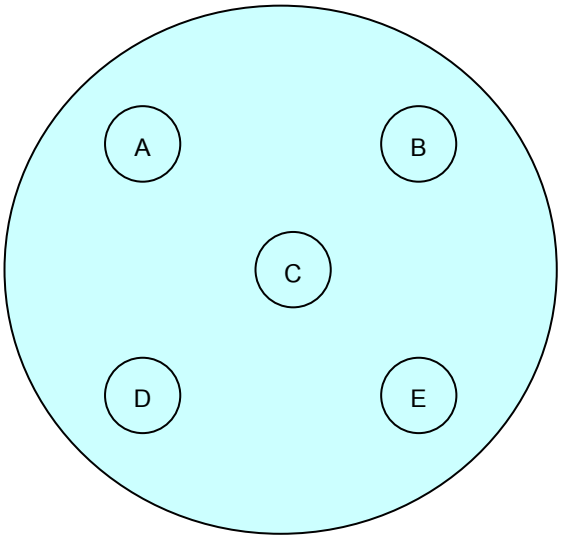


Fig 2 Disc diffusion assay: A=supernatant from M-MRS broth, B=M-MRS broth, C= supernatant from M-MRS broth adjust pH to 6.5, D= MRS broth, E= supernatant from MRS broth

Growth inhibition by Co-culture method

Five selected pathogenic bacteria (*S. iniae, S. agalactiae, F. indologenes, E.tarda* and *A.hydrophila)* were grown to lag phase in their suitable mediums. One hundred μl ($1x10^7$CFU/ml) of each pathogenic bacterium were inoculated in 10 ml of TSB with one hundred μl ($1x10^7$CFU/ml) of LGG.

After 24 hrs incubation at 37°C, the density of each pathogenic bacterium and LGG were estimated by using MRS agar for LGG and TSA for pathogenic bacteria. The results were expressed in percentage for each pathogen bacterium growth with LGG by co-culture method compared with each pathogen growth without LGG (control).

***In Vivo* study**

1. Fish, probiotic supplementation and *in vivo* experimental designs

One hundred tilapias, *Oreochromis niloticus*, 30-50 g body weight, were allowed to acclimatize for 7 days and were randomly placed in two 60-L tanks for the control and the probiotic groups. The tanks were filled with recycled water that was kept at 25˚-28˚ C, 5.8–6.8 ppm dissolved oxygen (DO) and 6.5–7.0 in pH throughout the experiment.

The probiotic bacterium, *L. rhamonsus*, was cultured in MRS broth at 37°C for 48 h, centrifuged and washed with sterile PBS 3 times. Bacterial pellets were measured in PBS and their densities were determined. Under sterile conditions, the bacteria were mixed into commercial dry pellets at the rate of 10^{10} CFU/g (Pirarat *et al*., 2006). The control fish were fed with the commercial dry pellets. They were fed approximately 1.5% of body weight once a day. At day 0, 14, 30 of feeding, the fish were weighed for the growth performance.

After feeding the fish with the probiotic for a month *S. iniae* strain II and one *S. agalactiae* strain I were selected to challenge the fish by injecting 0.2 mL ($2x10^8$ CFU/mL) of the bacterial solution intraperitoneally. Mortality and clinical signs were recorded for 14 days. Re-isolation and immunohistochemistry were used to confirm cause of the dead fish (Data not shown). This experiment was conducted three times.

For challenge test, relative percent survival (RPS) was calculated follow the equation (Yong *et al*., 2005):

RPS= [1- (Probiotic mortality/control mortality)] x 100

2. Growth parameters

For growth performance parameters, weight gain (%), specific growth rate and feed conversion ratio (FCR) were calculated by using the following equations (Yanbo and Zirong, 2005):

Weight gain (%) = 100 X (final mean body weight-initial mean body weight) / initial mean body weight

Specific growth rate = [(log n (final body weight) – log n (initial body weight) / days] x 100

Feed conversion ratio= feed intake (g) / Weight gain

3 Measurement of villous height, intraepithelial lymphocyte (IEL), acidophilic granulocyte and mucous cell

After 30 days of feeding, three parts of the intestine, the proximal part (after the pyloric part of the stomach to before the spiral part of the intestines), middle part (the spiral part of the intestines) and distal part (after spiral part of the intestines to 2 cm. before anus), from these fish in the probiotic and control groups were collected and fixed in 10% buffered formalin. Fixed tissues were processed according to standard histological techniques and tissue sections were stained with haematoxylin and eosin (H & E). For villous height measurement, 10 highest villi were selected per section. The villous length was measured from the villous tip to the bottom. An average of these 10 villi per section was expressed as the mean villous height for each section. For IEL, 10 highest villi which were selected to measure the villous height were given an arbitrary score from 0 to 3 based on the frequency and population number: 0 - none, 1 - mild, 2 - moderate and 3 - marked IEL. For acidophilic granulocytes, they were counted the whole tissue. For mucous cells, the fixed tissues were processed according to standard histological techniques and tissue sections were stained with combination of Alcian blue (AB) and periodic acid-Schiff reagent (PAS).Then 10 highest villi were selected and score base on frequency and population number.

4. Histopathology

For experimental challenge, six tissues (brain, spleen, head kidney, trunk kidney, liver and intestines) of three fish in each group were collected at 0, 3, 7 and 14 days post infection (DPI) and fixed in 10% buffered formalin for histopathology. The unexpected moribund fish in the control group were also sampled. Fixed tissues were processed according to standard histological techniques and tissue sections were stained with haematoxylin and eosin (H & E). The tissue sections were given an arbitrary score from 0 to 3 based on the frequency and severity of the lesions: 0, for no histopathological change; 1, mild; 2, moderate and 3 for severe histopathological change

5. Immunological aspects

5.1 Preparation of head kidney leukocytes

Head kidneys and blood were collected from 6 fish in each group at 14 days. The head kidneys were rinsed with RPMI1640 and separated through a 100 µm metal mesh into RPMI1640 medium using silicon tip to dislodge the leukocytes. Two ml of percoll solution (1.079 g/l) were added and spun at 350-400 g for 20 minutes at 4°C. The leukocytes were obtained from the interface and washed twice with RPMI1640 (10%FBS). The number of leukocytes was counted using a

haemocytometer with Tryphan blue inclusion. The experiment was continued upon the survival of 95 % of leukocytes for chemiluminescence assay. The blood samples were obtained by using syringes through the caudal vessels and centrifuged to make the sera for lysozyme and complement activity assay.

5.2 Phagocytic activity

Zymosan (0.5mg/ml) and leukocytes (1x 10^6 cells/ml), each 300 ul were added, and incubated for 30 minutes at room temperature. After incubation, the contents were washed with RPMI1640 (10%FBS). The 200 µl of mixtures were spun and placed on a clean glass slide using cytospin. Slides were stained with Giemsa's stain and observed for phagocytic activity as the percentage of phagocytising cells quantified from 200 phagocytic cells under a microscope. The phagocytic index was calculated from total number of ingested zymosan divided by number of leukocytes ingesting at least one zymosan. The experiment was repeated for 3 times and the values were analyzed statistically.

5.3 Chemiluminescence assay of leukocytes

The 1 x 10^7 cells/ml leukocytes used for this assay were incubated for three minutes at 28°C, the contents were spun down and the pellets were resuspended with 50 µl of RPMI-1640. The CLA (2-methyl-6-phenyl-3, 7-dihydroimidazo [1,2-a] pyrazine-3-one) dependent chemiluminescence was assayed in a lumi-counter (Microtech Nichion NU2500, Japan) by mixing 50 µl leukocytes, 100 µl CLA, 300 µl PBS (-) and 50µl phorbol 12-myristate 13-acetate (1µg/ml). The peak value was recorded and tested for statistical analysis.

5.4 Serum lysozyme activity

The blood samples were obtained by using syringes through the caudal vessels and centrifuged. Lysozyme activity in serum was assayed according to the method of Demers and Bayne (1997) based on the lysis of the lysozyme sensitive gram positive bacterium, *Micrococcus lysodeikticus* (Sigma). The change in turbidity was measured every 30 s for 5 min at 450 nm using a microplate reader.

5.5 Alternative complement activity (ACH50)

The alternative complement activity was determined following Yano (1992) by using rabbit red blood cells (RaRBC). The optical density of the supernatant was measured at 414 nm using a DU 640 spectrophotometer (Beckman Instruments Inc., California, USA). A lysis curve was obtained by

plotting the percentage of haemolysis against the volume of serum added. The volume yielding 50% haemolysis was determined and in turn used for assaying the complement activity of the sample (ACH50 value = units/ml).

5.6 Serum bactericidal activity

At 30 days after probiotic feeding and 7 days and 14 days post infection, serum of fish (6 each) were taken. One hundred µl ($1X10^7$ CFU/ml) of *Streptococcus iniae* was mixed with 100 µl of fish serum in each group for 1 hr. The number of *S. iniae* was estimated by preparing 10-fold serial dilution in peptone–saline dilution and spreading dilutions on tryptic soy agar (TSA).

5.7 Real Time RT-PCR

Total RNA was extracted from head kidneys using Trizol reagent. After the final wash with ethanol, the pellet was air-dried, dissolved in diethylpyrocarbonate (DEPC)-treated water and stored at 80 °C. The total RNA concentration and purity of the samples were measured. Primers for the TNF and IL-1 amplification have never been described elsewhere. For the TNF alpha, we used 5'-GCTGGAGGCCAATAAAATCA-3' and 5'-CCTTCGTCAGTCTCCAGCTC-3 for sense and anti-sense primers, which span the region from 489 to 827 on the TNF alpha coding sequence to amplify a band of 339 bp. For the IL-1, we used the sense 5'-TGCTGAGCACAGAATTCCAG-3' and the antisense 5'-GCTGTGGAGAAGAACCAAGC-3' primers, which span the region from 459 to 829 on the IL-1 coding sequence to amplify a band of 371-bp. L32 amplification was used as a control to normalize the amounts of input RNA. Real time RT-PCR was performed on MyCycler™ thermal cycler (Biorad, Japan). The RT-PCR reaction mixture (20 µl) contained 1x iQ™ SYBR Green Supermix (Bio-Rad, CA), 4 µl cDNA sample and 2 µl of the appropriate forward and reverse PCR primers. PCR conditions included an initial denaturation at 95 °C for 3 min, followed by a 40-cycle amplification consisting of denaturation at 95 °C for 15 s, annealing at 60 °C for 15 sec and extension at 72°C for 15 sec. As a control for each primer pair and each RNA sample, the cDNA synthesis reaction was carried out in the absence of reverse transcriptase in order to identify whether the RNA samples were contaminated by residual genomic DNA. The critical threshold cycle (C_t) was defined as the cycle in which fluorescence becomes detectable above the background fluorescence and is inversely proportional to the logarithm of the initial number of template molecules. A standard curve was plotted for each primer set with C_t values obtained from amplification of known quantities of *S. mutans* cDNA. The standard curves were used for transformation of the C_t values to the relative number of cDNA molecules.

Statistical analysis

Data were expressed as a mean ± S.D. and were evaluated using one-way ANOVA followed by the Bonferroni-type multiple t-test. For each histopathology score, the data were subjected to analysis of variance by using a nonparametric Mann–Whitney test. All tests used a significant difference level of $P < 0.05$.

Results

In Vitro Antimicrobial activity Results

Agar spot test

The agar spot test in both M-MRS and TSA agar showed that all strains of *S. iniae* and *S. agalactiae* had inhibition zones, especially on M-MRS agar (Table1). The widest inhibition zone on M-MRS agar was found in *S. iniae* IV and the widest inhibition zone on TSA was found in *S. iniae* II. There was no inhibition zone in any strains of *S. iniae* and *S. agalactiae* by killed probiotic bacteria.

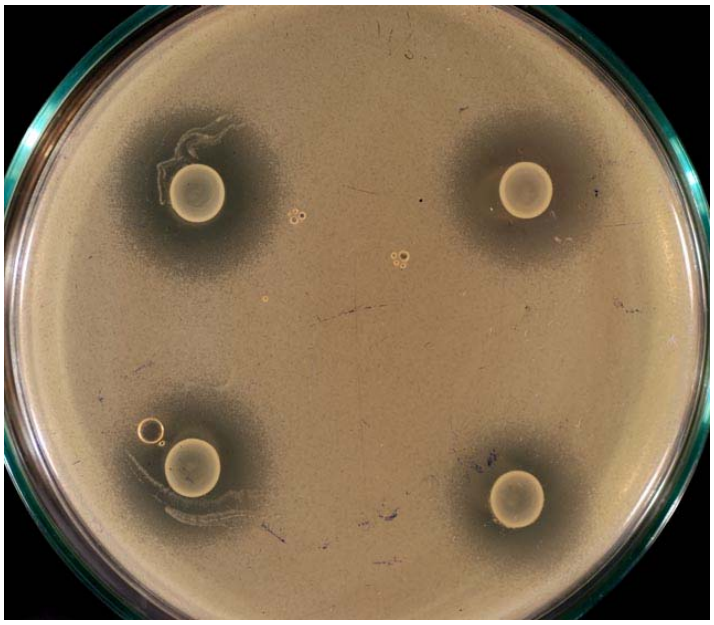


Fig. 1 Agar spot test on M-MRS agar with *S. iniae* IV

Disc diffusion assay

Cell-free supernatant from 48 h culture of *L.rhamnosus* was able to inhibit the growth of all 4 strains of *S. iniae* and 2 strains of *S. agalactiae.* Strain II of *S. iniae* was the most susceptible. (Table 1)

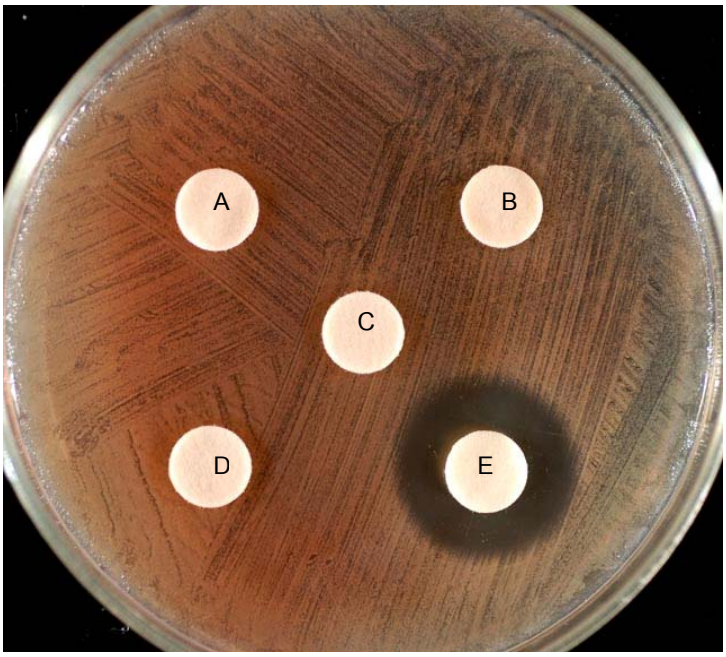


Fig. 2 Disc diffusion assay A=supernatant from M-MRS broth, B=supernatant from M-MRS broth adjust pH to 6.5, C=M-MRS broth, D=MRS broth, E=supernatant from MRS broth

	Agar spot test				**Disc diffusion**	
	M-MRS		TSA			
	inhibition zone	SD	inhibition zone	SD	inhibition zone	SD
S. iniae I	0.58	0.46	0.25	0.06	0.25	0.1
S. iniae II	0.52	0.46	0.73	0.21	0.8	0.2
S. iniae III	0.52	0.07	0.25	0.14	0.25	0.13
S. iniae IV	1.2	0.96	0.23	0.05	0.23	0.05
S. agalactiae I	0.9	0.23	0.15	0.05	0.15	0.06
S. agalactiae II	0.2	0.07	0.28	0.09	0.275	0.1

Table 1 Inhibition zone of agar spot test and disc diffusion assay (n = 8)

Co-culture

After having incubated, each pathogen with LGG for 24 hrs, the growth of *S. iniae* and *E. tarda* was significantly lower than in control.

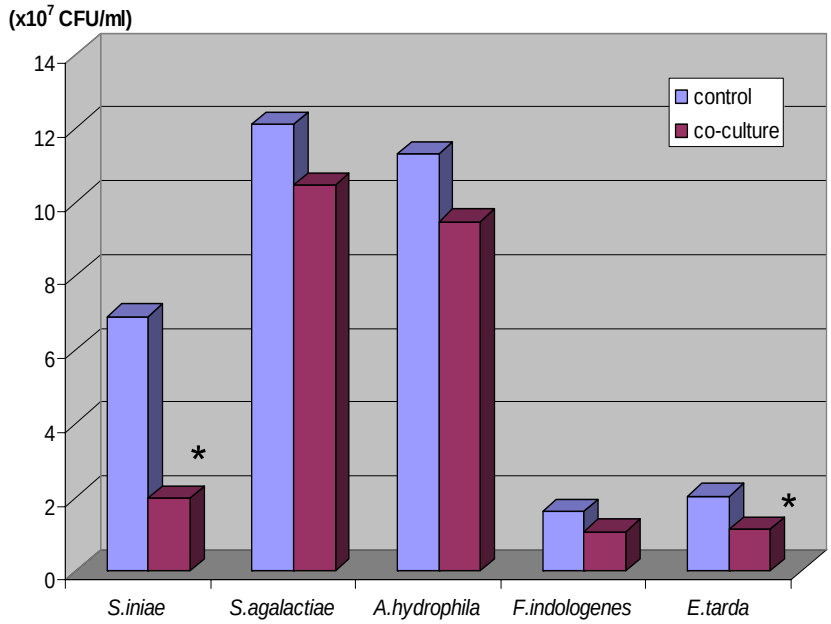


Fig. 3 Effect of probiotic on the growth of pathogens bacteria (n=6)

Growth performance

Data on the growth performances of the tilapias, including weight gain (%), specific growth rate and feed conversion ratio (FCR) of the tilapias are shown in Fig. 4. The probiotic group showed higher weight gain (%), specific growth rate and feed conversion ratio than the control in the first and second phases of feeding.

Growth performance	Control	Probiotic
Initial mean body weight	22.77	22.35
Mean body weight (14 d)	26.79	26.85
Mean body weight (30 d)	30.03	30.52
Weight gain (%) 14 days	15.95	18.65
Weight gain (%) 30 days	23.55	24.60
Specific growth rate (14 d)	1.04	1.13
Specific growth rate (30 d)	0.89	1.01
Feed conversion ratio	1.46	1.24

Table 2 Growth performance after probiotic feeding (n=50)

Measurement of villous height

The villous height in proximal intestine was the greatest followed by the middle part and the distal part. In the proximal, the middle and the distal intestine, the villus height of the probiotic group was significantly greater than that of the control group (P<0.05).

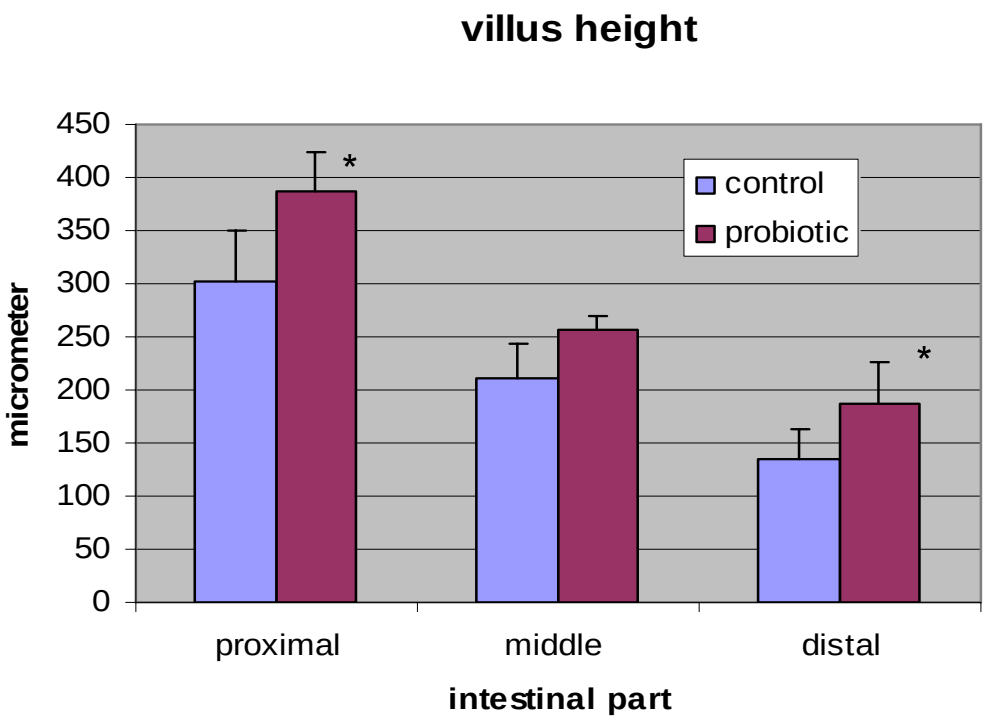


Fig. 4 Villus height between probiotic and control group probiotic feeding for 30 days. (* significant difference among the groups, P < 0.05, N = 10)

Intraepithelial lymphocyte and acidophilic granulocyte

The population of intraepithelial lymphocyte in the control group was gradually increasing from the proximal part to the distal part of intestine while in the probiotic group the population with intraepithelial lymphocyte was remarkable in all parts. In the proximal and the middle parts of the intestine, the probiotic group had significantly higher population with intraepithelial lymphocyte than the control group(fig 5).

The population with acidophilic granulocyte was greater in the probiotic group in proximal part and distal part of the intestine. (fig 6)

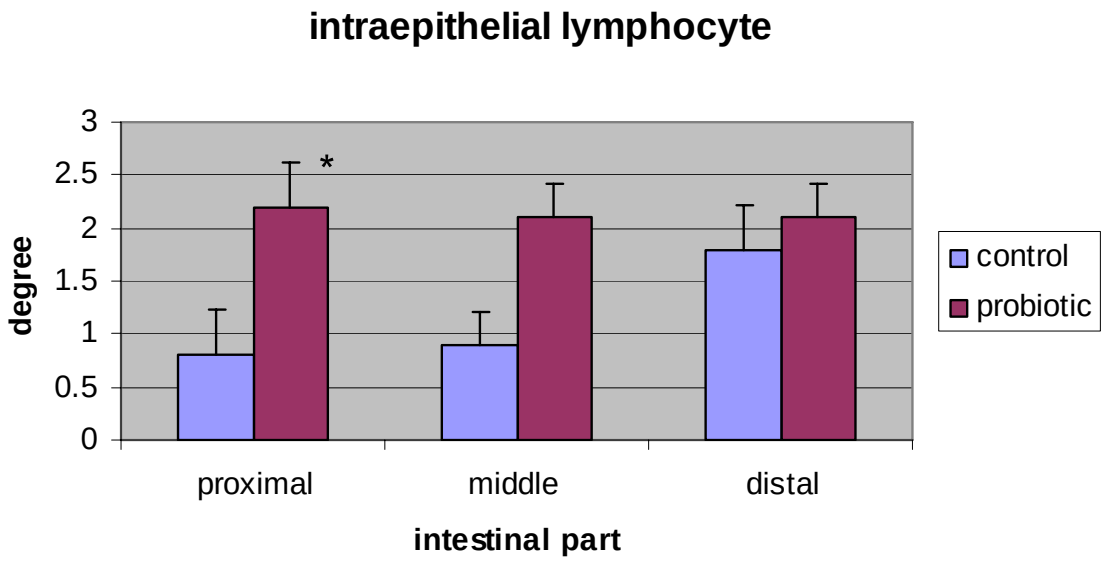


Fig. 5 Intestinal intraepithelial lymphocyte after probiotic feeding for 30 days (* significant difference among the groups, $P < 0.05$, $N = 10$)

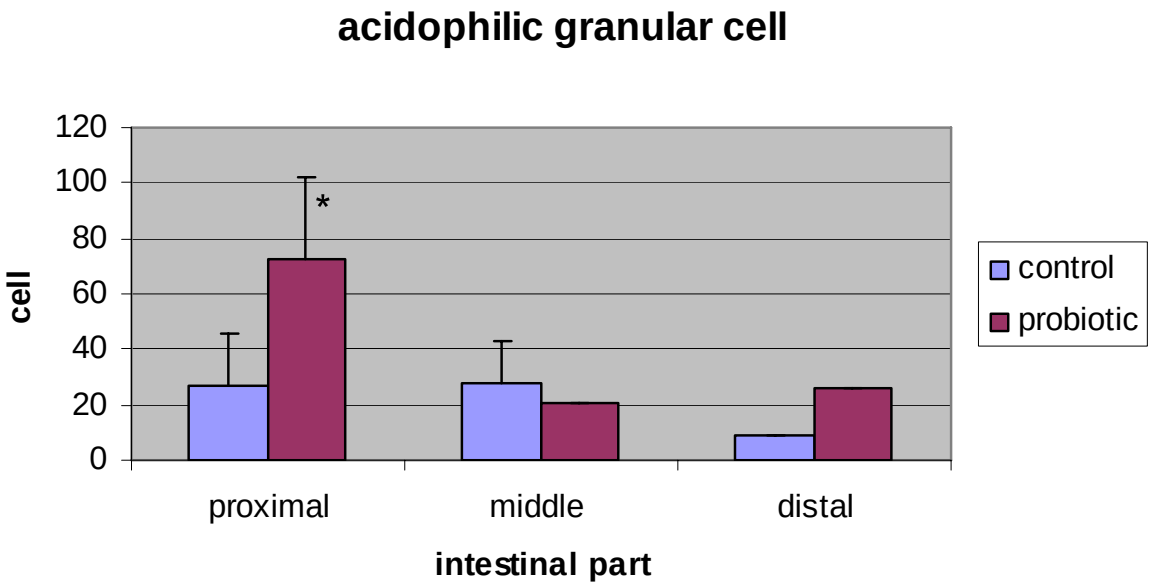


Fig. 6 Intestinal acidophilic granular cell after probiotic feeding for 30 days

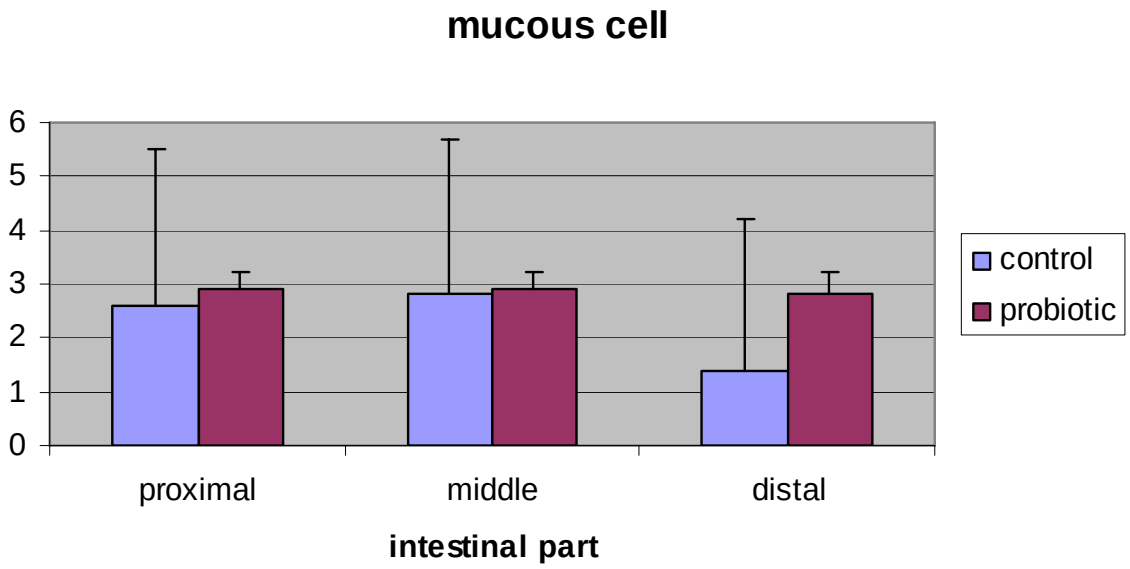
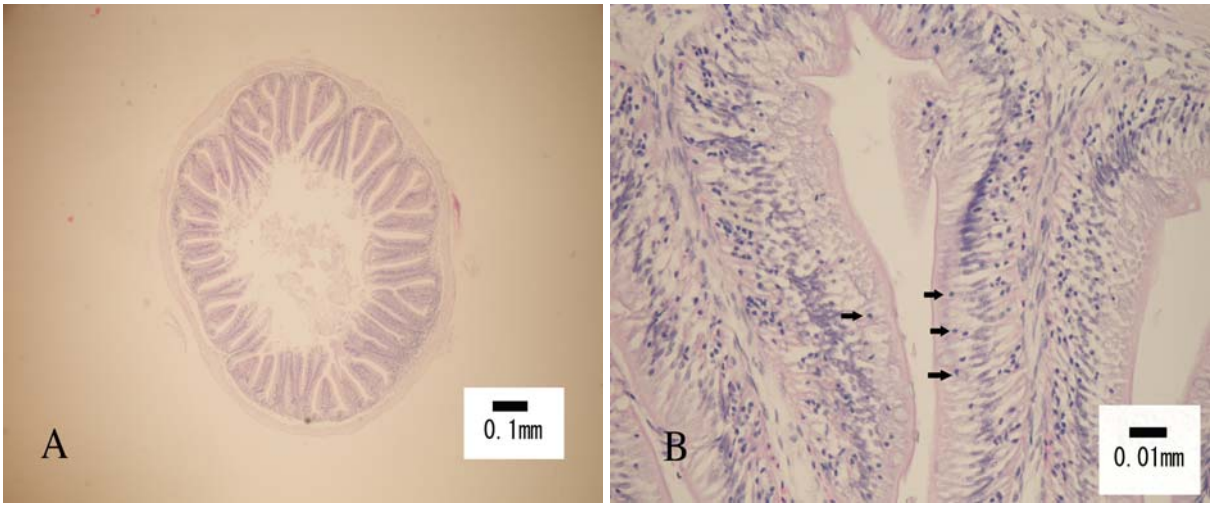


Fig. 7 Intestinal mucous cell after probiotic feeding for 30 days

Intestine mucous secretion cells

The population of mucous cells in the control group decreased from the proximal part to the distal part, while in the probiotic group the population with mucous cells increased from the proximal part to the distal part. When the same parts of the intestine between the probiotic and the control groups were compared, in the distal part, the population of mucous cells in the probiotic group was greater.



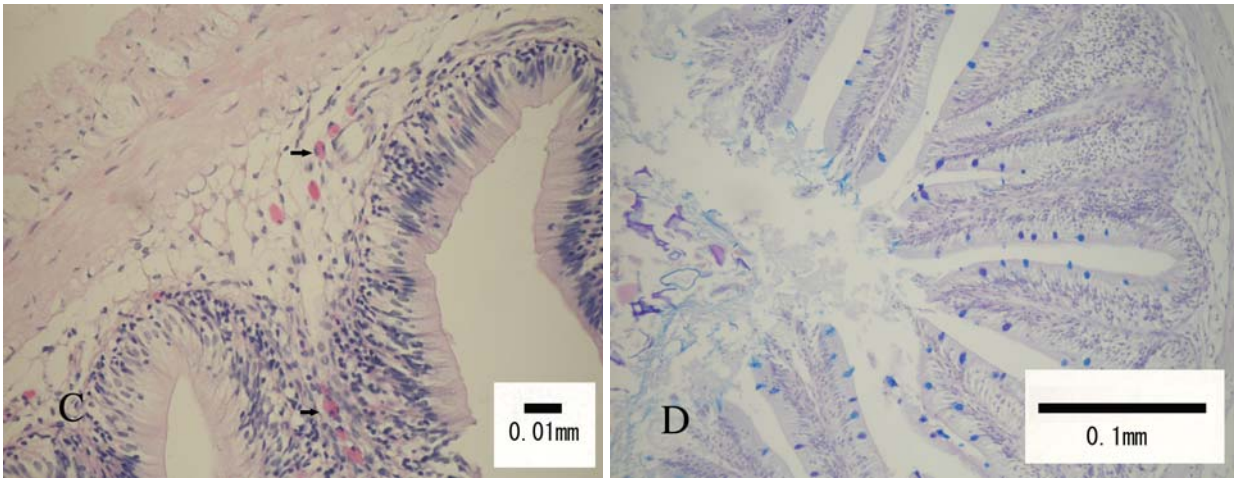


Fig. 8 Histology of proximal part of intestine of probiotic group (A), arrow intraepithelial lymphocyte H&E (B), arrow acidophilic granulocyte H&E (C), mucous cell combination of PAS and AB (D)

S. iniae and *S. agalactiae* challenge test

The initial mortality of fish in the *S. iniae* challenge trial began at 2 DPI in the control group and on 4 DPI in the probiotic group. Mortality still occurred until 12 DPI in the control and probiotic groups. The highest mortality was recorded at 6 DPI in the control group and at12 DPI in the probiotic group. The relative percent survival (RPS) in the probiotic group was 62.5. The cumulative mortality was significantly lower in the probiotic group (12%) than in the control group (32%). While challenging with *S. agalactiae*, the first mortality began at 1 DPI in the control and probiotic groups. Mortality still occurred until 12 DPI in the control group and until 8 DPI in the probiotic group. The highest mortality was recorded on 3 DPI in the control group and at 2 DPI in the probiotic group. The relative percent survival (RPS) in the probiotic group was 46.43. The cumulative mortality was significantly lower in the probiotic group (16%) than in the control group (34.7%).

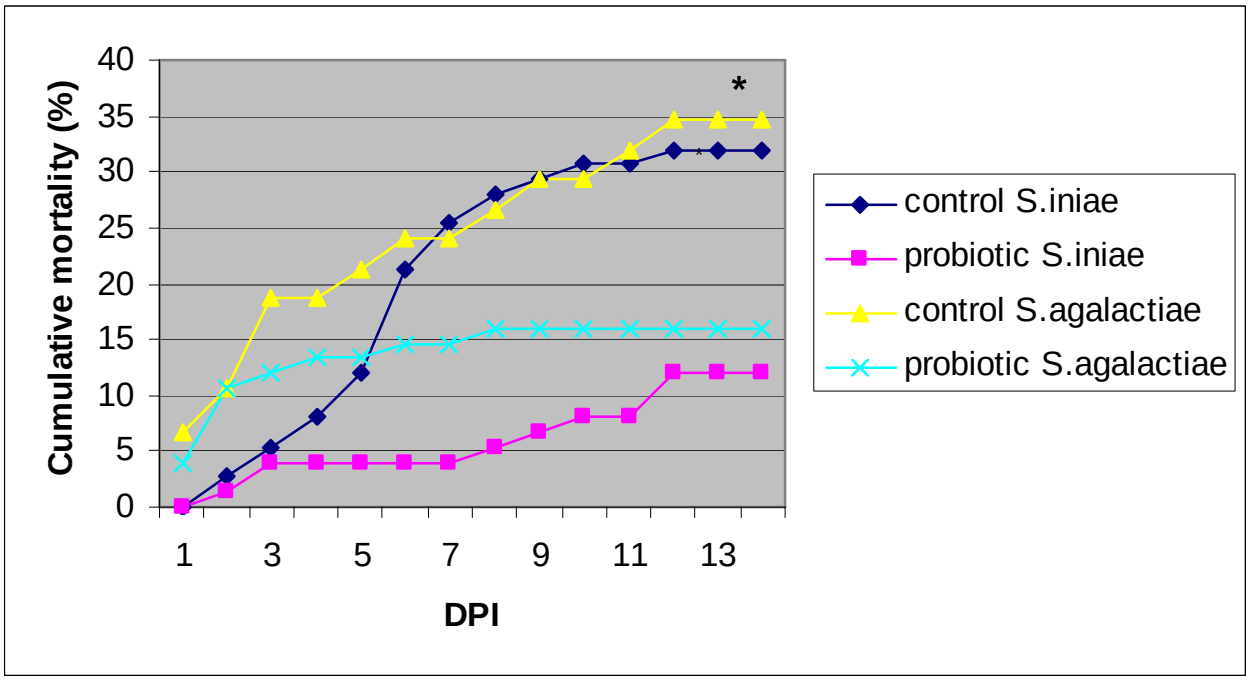


Fig. 9 Average cumulative mortality.

* Significant statistical difference among the groups ($P < 0.05$).

Histopathology

The *S. iniae* and *S. agalactiae* challenges caused many kinds of alterations in various organs in both the control and the probiotic groups (Table 3). In the head kidneys and spleens, the increase of melano-macrophage center was very noticeable in both the control and probiotic groups. In the head kidney, at the beginning (3DPI) of the challenge tests, there was found an increase of the melano-macrophage center, which was still higher on 7 DPI and decreased in later stages of infection (14DPI) except in the control group challenged with *S. agalactiae*. In the moribund fish of both challenges, there was also an increase, but significantly in a lower degree when compared with the other groups. In the same way, the spleens also showed an increase of melano-macrophage center after being challenged. There were remarkable lesions of haemolysis of red blood cells in the head kidneys in the probiotic and control groups, but both of the probiotic groups incline to decrease this lesion. The moribund fish of both challenges tend to have more severe lesions than the other groups particularly when challenged with *S. agalactiae*.

In the trunk kidneys, histopathological changes were found more in early stages of infection (before 7 DPI). The renal tubular degeneration characterized by hyaline deposition in tubular cells with the nucleus displaced to the side occasionally with renal tubular necrosis and calcification of renal tubules was discovered in all groups. In the livers, fatty degeneration was found in a low

extent and glycogen degeneration was found in a high extent in all four groups. The brains from the probiotic groups of both challenges showed a lower degree of meningitis than from the control groups, while the degree of meningitis in the moribund fish was more severe.

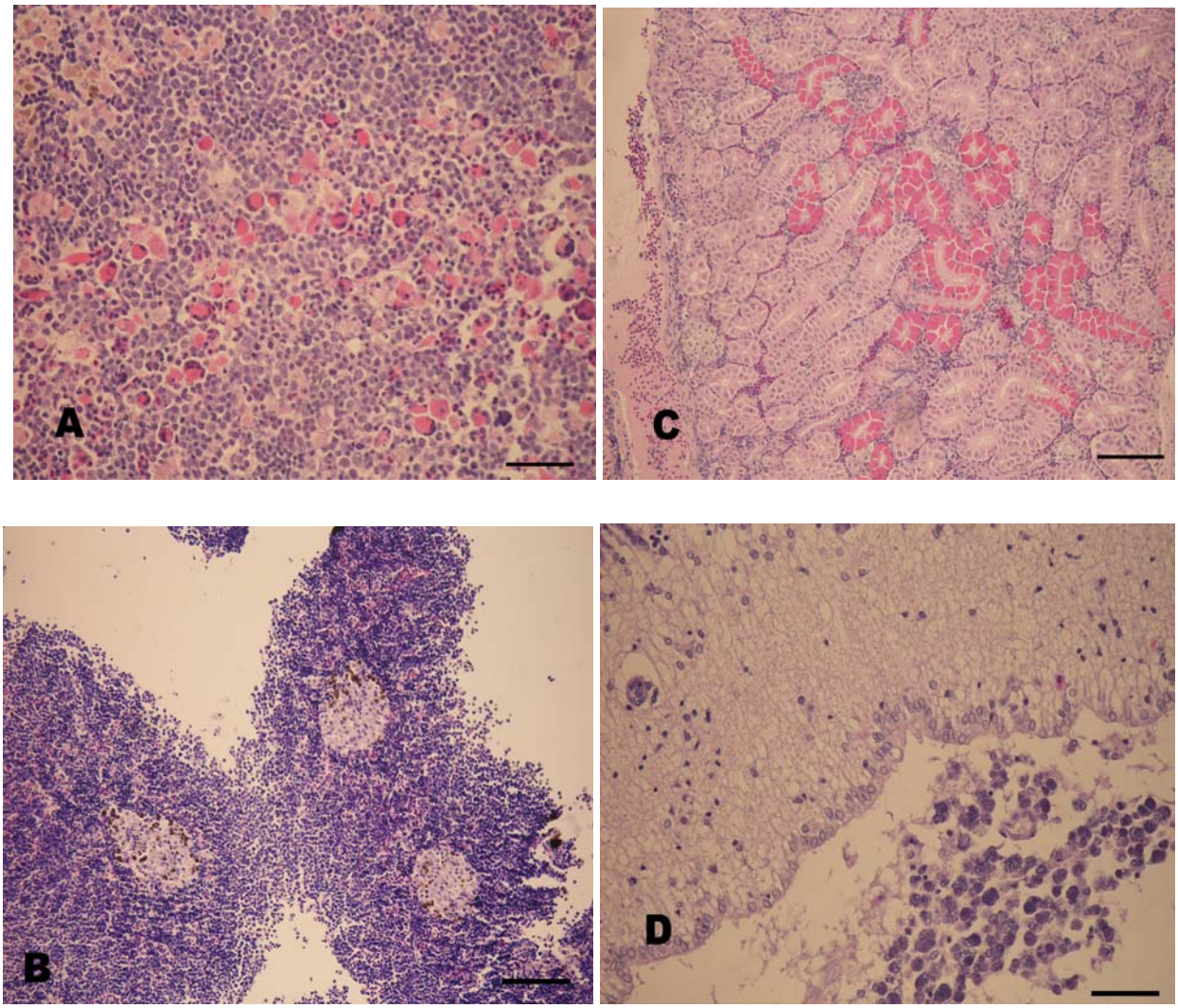


Fig 9A Heamolysis in head kidney from moribund fish challenged with *S. agarlactiae* Bar=125 µm

Fig 9B Granuloma-like lesion in probiotic group challenge with *S. agarlactiae*, Bar= 12.5 µm

Fig 9C Tubular degeneration from control group challenged with *S. agalactiae* (7 DPI), Bar=125 µm

Fig 9D Meningitis in moribund fish challenged with *S. agalactiae* Bar= 250 µm

	Control SI				Probiotic SI				Control SA				Probiotic SA				M.SI	M.SA
	0	3	7	14	0	3	7	14	0	3	7	14	0	3	7	14		
Head kidney																		
MMC	0.33	0.76	2	1	0.33	1.67	2	0.33	0.33	1.33	1.67	2	0.33	1	2.33	2	1	1
Heamolysis	0	1.33	2	1.33	0	0.67	1.67	2	0	1	1	2	0	0.67	1.33	1.67	2	2.33
Spleen																		
MMC	1	1	1.33	2.67	1	1.33	2.87	1.33	1	1.33	2.67	1.33	1	1.67	2.33	3	2	2.33
Trunk kidney																		
T. degeneration	0	1.33	2	1.5	0	1.17	1.83	0.83	0	1.83	2	1	0	0.83	3	2.33	2	2.76
T. regeneration	0	0	1.33	1	0	0	0.33	1	0	0	0.67	0.67	0	0	0	1.33	0	0
Calcification	0	0	1	1.17	0	0.17	1	1.33	0	1.33	0.5	0	0	0.17	0.5	0	0	0
Brain																		
Meningitis	0	1	1	1	0	1	0.67	0.67	0	0.67	1	1.33	0	0.33	0.33	0.33	2	2
Liver																		
Fatty degeneration	0.33	0.67	0	0	0	1	1	1.67	0.33	0	0	0	0	1	0.67	1	2	1.67
Glycogen degeneration	0	2	1.67	2	0	1.67	2	1.67	0	1.67	1.67	2.33	0	3	2	2	1	2.33

Table 3 Histopathology scores of tilapias infected with *S. iniae* and *S. agalactiae*

SI=*S. iniae*, Sa=*S. agalactiae*, M.= moribund fish, T.= tubular

Phagocytic assay

There was no difference in phagocytic activity and phagocytic index between the probiotic and control group after 30 days *Lactobacillus rhamnosus* supplementation

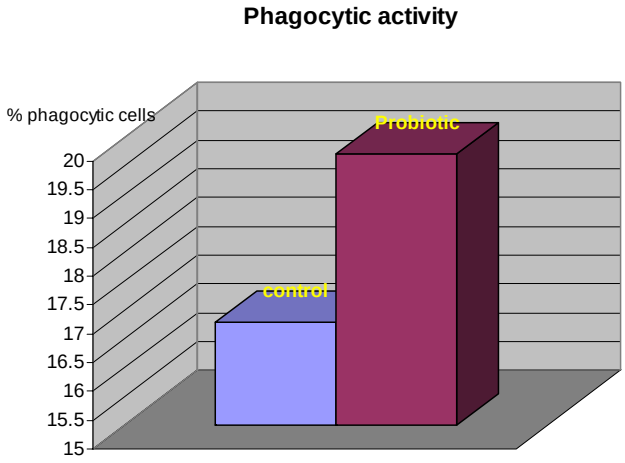


Fig. 10 Phagocytic activity of head kidney leukocytes after probiotic feeding

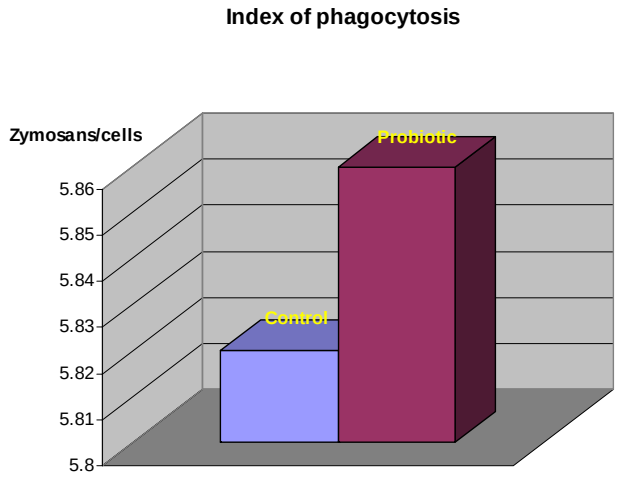


Fig. 11 Phagocytic index of head kidney leukocytes after probiotic feeding

Chemiluminescence activity

The chemiluminescence activity of head kidney leukocytes in probiotic group was significantly greater than in control group.

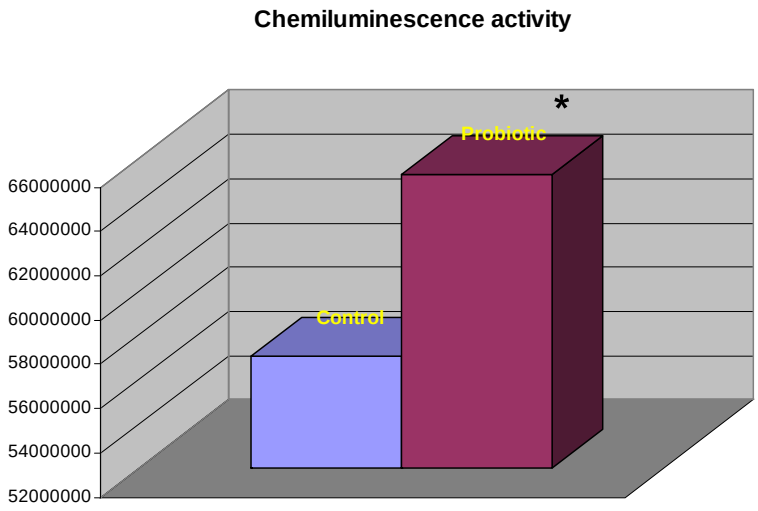


Fig. 12 Chemiluminescence activity response of head kidney leukocytes after probiotic feeding

Alternative complement activity

ACH50 activity was significantly higher in the probiotic supplemented group than in the control group. The probiotic fish maintained the high ACH50 activity throughout the test period.

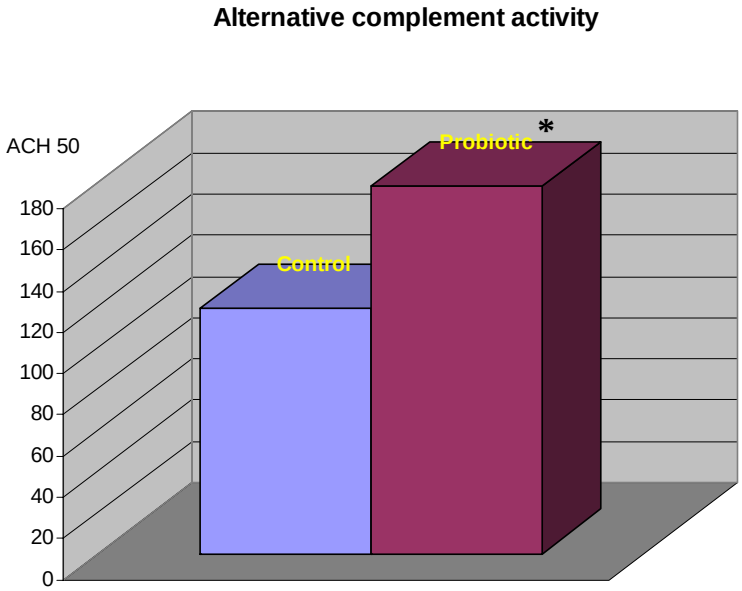


Fig.13 Serum complement activity of tilapia after probiotic feeding

Serum lysozyme activity

Serum lysozyme activity was rather high in both control and probiotic group. Slightly increase in serum lysozyme activity was observed in probiotic group.

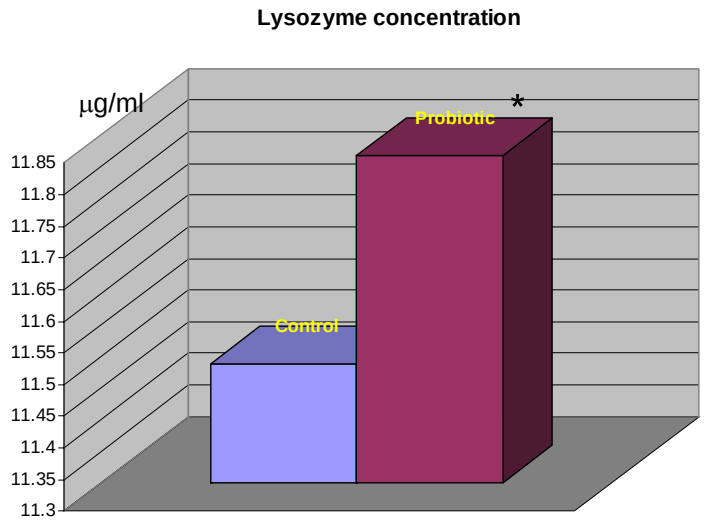


Fig.14 Serum lysozyme concentration of tilapia after probiotic feeding

Serum bactericidal activity

Fish serum from 3, 7, and 14 DPI was effective in probiotic and in control groups. On 7 and 14 DPI, was significantly more effective in probiotic group than in control group. (Fig.1)

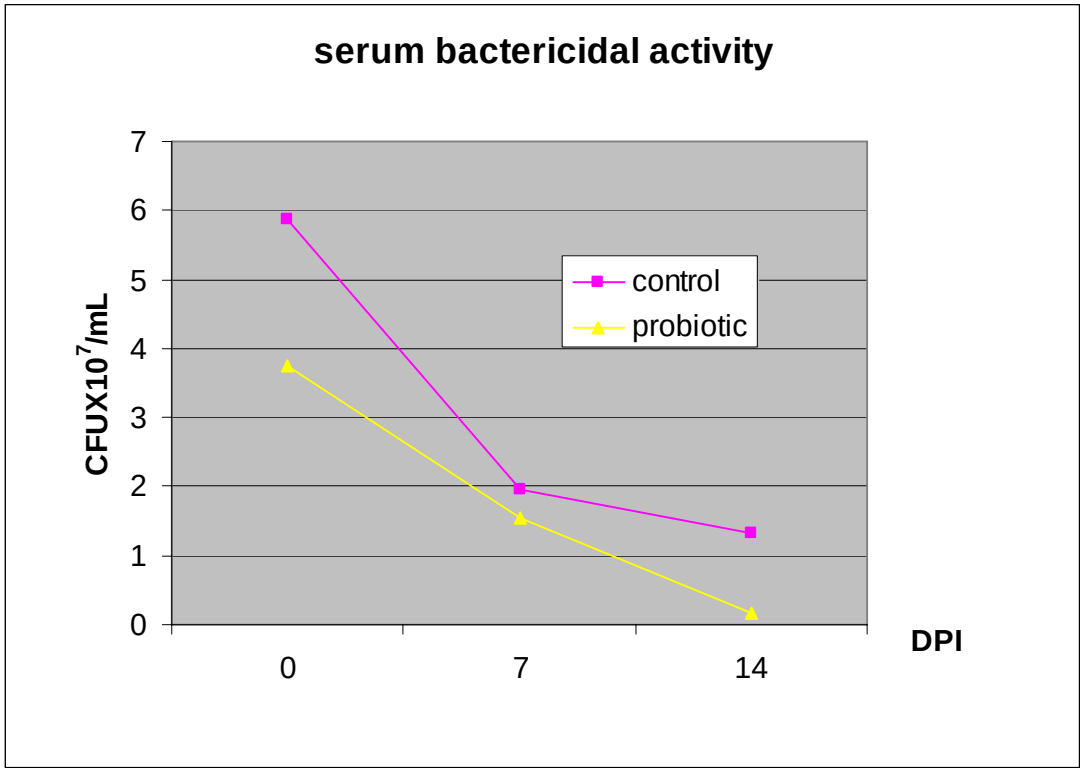


Fig.15 Serum bactericidal activity of tilapia after probiotic feeding

Real Time RT-PCR analysis

The relative expression of IL-1 and TNF alpha gene was significantly higher in probiotic fish when compensated with that of the L-32 genes in head kidney. However, the relative expression of IL-1 and TNF alpha gene in spleen seems to showed insignificant difference between the probiotic and control fish.

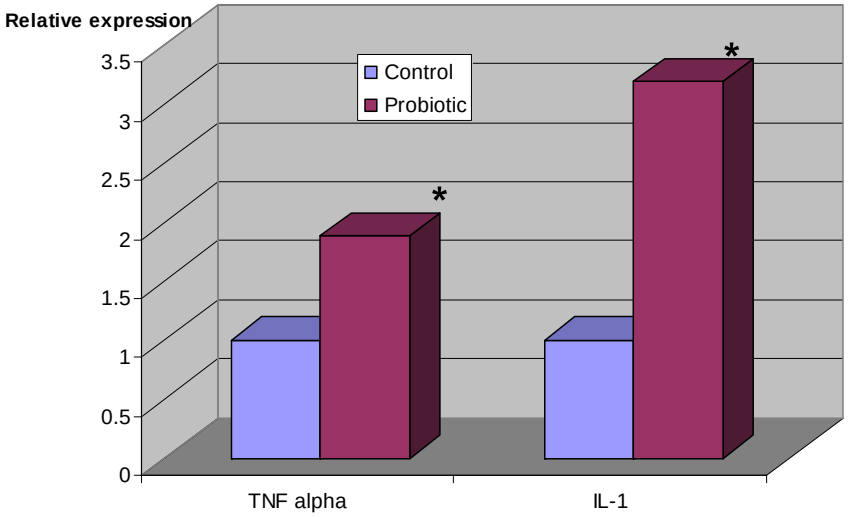


Fig.16 The relative expression of IL-1 and TNF alpha gene in head kidney after probiotic feeding

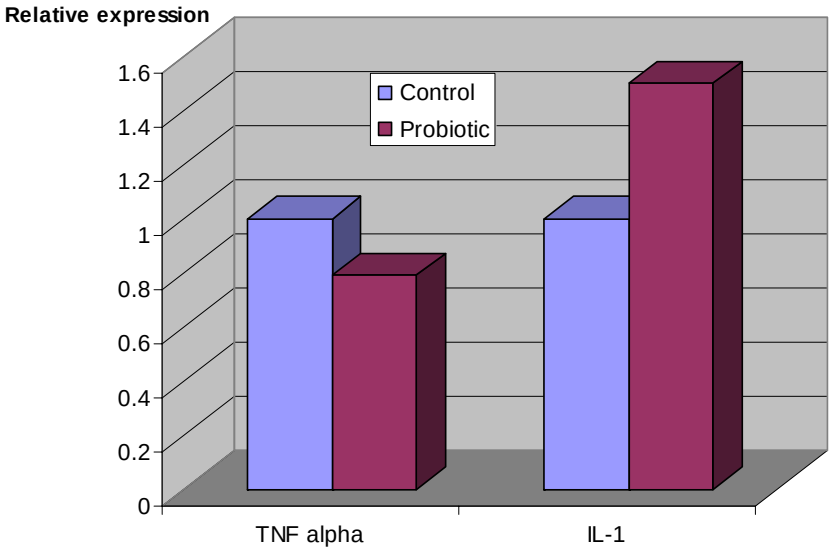


Fig.17 The relative expression of IL-1 and TNF alpha gene in spleen after probiotic feeding

Discussion

The probiotic-supplemented diet groups resulted in better growth performance and lower FCR than the control groups, suggesting that the addition of probiotic can improve the growth performance and feed utilization. Several experiments studied using *Lactobacillus* sp. as the dietary supplement to increase growth performance in fish (Suzer *et al*., 2008; Aly *et al.,* 2008). The improvement in growth and feed utilization in the probiotic-supplemented group could be induced by the effects of probiotic action, including the maintenance of normal intestinal microflora, improving nutrition by detoxifying the notorious compounds in feeds together with denaturing the potentially indigestible components in the diet by hydrolytic enzymes, including amylases and proteases and producing vitamins such as biotin and vitamin B12 (Balcazar *et al*., 2006; Fuller, 1989; Planas *et al*., 2004; Suzer *et al*., 2008).In addition, the morphology of the villi is the important thing that can point out the growth performance. The increase in the length of the villi implies an increase of surface area for greater absorption of available nutrients (Caspary, 1992). In this present study, the probiotic group had longer in villus height in all parts of the intestines, significantly in the foregut and midgut. This corresponds with the longer villi in chickens and turkeys treated with *L. reuteri* (Dunham *et al*., 1993). Besides, probiotics have proven to induce gut epithelium cell proliferation in rats (Ichikawa *et al*., 1999). The possible mechanism could be that after the probiotic bacteria are transited to the stomach, they germinate in the intestines and use a large amount of sugar for their growth, producing substance that contribute to make short chain fatty acids, which might play an important role in increasing the villus height (Pelicano *et al*., 2005). Short chain fatty acid particularly butyric acid is the main energy source for colonic epithelium cells and able to stimulate the release of gastrointestinal peptide or growth factors which may affect cell proliferation. (Blottiere *et al*., 2003)

The present study on the agar spot test showed the efficacy of the antimicrobial activity of *L. rhamnosus* over all strains of *S. iniae* and *S. agalatiae*. The agar spot test using killed probiotic bacteria, which showed no inhibition zones, clearly showed that only the metabolite products, not the cells of probiotic bacteria, are involved in the growth inhibition of *S. iniae* and *S. agalatiae*. Lactic acid bacteria are known for their ability to produce inhibitory substance such as hydrogen peroxide, organic acid and bacteriocin-like products, which are antimicrobials (De Vuyst and Leroy, 2007). Moreover from result of disc diffusion assay which showed the inhibition zone only when applied the supernatant from MRS broth and it may suggest that antimicrobial substances could be from lactic acid bacteria because the main metabolite product from MRS while using M-MRS broth decrease producing lactic acid from

L.rhamnosus. In addition, the result from the agar spot test on TSA, which is not suitable media for lactic acid, might suggest that even in an unfavorable environment *L. rhamnosus* still can produce antimicrobial substrates.

In aquatic animals, there are many studies reporting about probiotics in modulating immunity such as inducing of proinflammatory cytokines, stimulating the activity of natural killer cells, increasing mucosal and systemic antibody production, activating phagocytic activity and increasing lysozyme and complement activity (Nikoskelainen *et al.*, 2003, Pirarat *et al.*, 2006). The focus of this study is in gut mucosal immunity, an important part in protecting host from pathogen. The gut mucosal lymphoid tissue in fish is different from mammals' because the former lacks of Payer's patch and antigent-transporting M cells. It normally is composed of organized lymphoid cell, macrophages and granulocytes. IEL, the component of gut association lymphoid tissue, plays a major role in mucosal defense mechanisms against intraluminal foreign antigens(Kiristioglu *et al.*, 2002). In the histological survey of this study, the population of IEL in the probiotic group was significantly higher than in the control group in all parts of the intestine. The population of acidophilic granulocyte in the probiotic group was significantly higher in proximal and distal parts when compared with the control group. Although acidophilic granulocyte is a response of inflammation but from the histology in both the probiotic and the control groups there was absence of tissue damage and no evidence of edema and vasodilation. Histological finding supported the safety of probiotic as dietary supplement. This result is similar to the previous studies in European sea bass treated with *L. delbruekii*(Picchietti *et al.*, 2009) and also in seabream treated with *L. fructivorans* and *L. plantarum*(Elbal *et al.*, 2004). The higher of intraepithelial lymphocyte and the higher of acidophilic granulocyte in probiotic group might point out that administration probiotic affect intestinal immune cells.

The main functions of intestinal mucous cells are producing and secreting mucous gel which helps in lubricating, immobilizing enzyme, protecting the mucosal surface from exogenous and endogenous such as bile salts and trapping pathogenic bacteria and parasites. The mucous cell in control group scattered throughout the intestine but there no part is abundance clear which correlated with previously study in tilapia(A. M. Gargiulo, 1998). On the other hand, the more numerous of mucous cell in distal part were reported in other fish. In probiotic group, there is the more frequency in finding mucous cell than control group in all part of the intestine particularly in distal part. This comparable with the study in turkey with feeding probiotic (Rahimi *et al.*, 2009).

The efficacy of *L. rhamnosus* was determined from the *in vivo* model. The relative percent survival rate (RPS) in the probiotic group challenged with *S. iniae* is 62.5, which indicates that the probiotic could protect the fish from infection (according to European Pharmacopeia). Although, it gave an unsatisfactory effect in the *S. agalactiae* challenged groups (RPS is 46.43), the mortality tended to decrease in the probiotic group when compared with the infected control group. From the previous studies, *L. rhamnosus* has been proven for its potential to control infection from *Aeromonas salmonicida*, *Vibrio anguillarum* and *Flavobacterium psychrophilum* in rainbow trouts and turbots (Nikoskelainen *et al*., 2001). However, it is not clear how probiotics are involved this response. Panigrahi *et al,* 2004 studied on *L. rhamnosus* JMC1136 and found that it can stimulate non specific immune like phagocytosis, lysozyme and complement activities in rainbow trout. Another study showed similar results of *L. rhamnosus* enhancing the alternative complement, enabling phagocytic cell aggregation and increasing phagocytic activity (Pirarat *et al*., 2006). On histopathology in this study both challenges with *S. iniae* and *S. agalectiae* gave the same evidence of melano-macrophage centers in the head kidneys and spleens. In early stages of infection (before 7DPI), the probiotic-dietary supplement group had a higher degree of melano-macrophage centers than the control group. The melano-macrophage center in fish enable the functions of macrophages in domestic animals, phagocytes foreign materials, including infectious agents (Agius and Roberts, 2003). From histopathology, increasing of melano-macrophage center in head kidney both of probiotic groups could assume that using probiotic as a dietary supplement can enhance the immune response.

This study revealed the effect of feeding *Lactobacillus rhamnosus*-supplemented diets to tilapia, on some key immune parameters involved with the gut-associated lymphoid tissue at the mucosal level as well as the humoral and cellular immunity at the systemic level. In innate humoral response, complement activation play an important role in phagocytosis through chemotaxis and opsonization of phagocytic cells. In this study, the higher complement level as well as the enhanced phagocytosis and killing ability of head kidney leukocytes were correspondingly with the TNF alpha and IL1 expression in the probiotic supplemented fish. It might be noted that induction of cellular and humoral immune response via these cytokine expression might be generated by *Lactobacillus rhamnosus*-supplemented diet. Additional evidence exists in turbots where feeding of nucleotide-supplemented diets increased the expression of pro-inflammatory cytokines including IL-1 and TNF (Low et al., 2003 aquaculture). The result is in agreement with another report on rainbow trout where the

complement activity and IL-1 beta gene expression was enhanced upon feeding *Enterococcus faecium* supplemented diet (panigrahi et al., 2007)

All the results clearly suggest that *L. rhamnosus*, a human-derive probiotic, can be used in tilapia without negative effect in its intestine and give the advantage in promoting growth performance, improving intestinal mucosal immunity and intestinal structure.

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Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1 ผลงานที่คาดว่าจะตีพิมพ์ในวารสารวิชาการนานาชาติ

1.1 *In Vitro* efficacy of human-derived probiotic, *Lactobacilus rhamnosus* against pathogenic bacteria of fish and frog by Pirarat et al. (submitted to Thai Journal of Veterinary Medicine) (ภาคผนวก 1)

1.2 Immune responses in nile tilapia (*Oreochromis niloticus*) induced by *Lactobacillus rhamnosus* GG by Pirarat et al. (manuscript in preparation to Journal of Fish and Shellfish Immunity)

1.3 Efficacy of *Lactobacillus rhamnosus GG* against streptococcosis in tilapia (*Oreochromis niloticus*) by pirarat et al. (manuscript in preparation to Journal of Veterinary microbiology)

2 การนำผลงานวิจัยไปใช้ประโยชน์

2.1 เชิงวิชาการโดยนำองค์ความรู้ที่ได้จากการศึกษาเพื่อใช้ในการเรียนการสอนโครงการพิเศษ รหัสวิชา 3100610 ระดับปริญญาตรีคณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย เรื่อง The efficacy of *Lactobacillus rhamnosus* GG against Streptococcosis in tilapias (*Oreochromis niloticus*): Growth performance and protective effects (ภาคผนวก 2) และการศึกษาในระดับบัณฑิตศึกษาที่เกี่ยวข้องกับพยาธิชีววิทยาทางด้านสัตว์น้ำและสัตว์ป่า

- 2.2 เชิงสาธารณะ การสร้างเครือข่ายความร่วมมือ สร้างกระแสความสนใจในวงกว้างทั้งในต่างประเทศและในประเทศ โดยการบรรยายพิเศษในหัวข้อ โปรไบโอติกกับการเลี้ยงปลานิลให้แก่เกษตรกร นักวิชาการประมงและบุคคลทั่วไป (งานประชุมสัมมนาวิชาการ เรื่อง รู้ทันปัญหาสุขภาพและการเลี้ยงปลานิล วันที่ 2 ตุลาคม 2551 อาคารสถาบันวิจัย 3 จุฬาลงกรณ์มหาวิทยาลัย และวิทยากรบรรยายพิเศษ Application of Fish Vaccine in Aquaculture, 21st-24th July 2008 in Ho Chi Minh City, Vietnam)

2.3 ผลงานอื่นได้แก่ ผลงานตีพิมพ์ในหนังสือรวบรวมบทความวิชาการระดับนานาชาติ จำนวน 3 เรื่อง

- The efficacy of a human-derived probiotic, *Lactobacillus rhamnosus* GG, against *Streptococcus iniae* in tilapias (*Oreochromis niloticus*). 5th World Fisheries Congress, 2008. Yokohama, Japan (ภาคผนวก 3)

- *Lactobacillus rhamnosus* GG, a potential human-derived probiotic candidate for tilapia (*Oreochromis niloticus*) culture 2008. The 15th Congress of the Federation of Asian Veterinary Associations FAVA-OIE Joint Symposium of Emerging Diseases, Bangkok, Thailand. (ภาคผนวก 4)

- *In Vitro* efficacy of Human-Derived Probiotic, *Lactobacilus rhamnosus* Against Pathogenic Bacteria of Fish and Frog 2008. The 15th Congress of the Federation of Asian Veterinary Associations FAVA-OIE Joint Symposium of Emerging Diseases, Bangkok, Thailand. (ภาคผนวก 5)

ภาคผนวก

The efficacy of a human-derived probiotic, *Lactobacillus rhamnosus* GG, against *Streptococcus iniae* in tilapias (*Oreochromis niloticus*).

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Abstract

Probiotic supplementation is now being focused as an alternative method to control fish diseases. This study investigates the protective effects of *L. rhamnosus*-supplemented diet against *Streptococcus iniae* after intraperitoneal challenging at a concentration of $2x10^8$ CFU/fish. An *in vitro* study on antimicrobial activity using agar spot test and disc diffusion showed that *L. rhamnosus* strongly inhibited the growth of *S. iniae* in all 8 strains. In the *in vivo* study, Cumulative mortality was lower in probiotic-supplemented fish (11.4%) than in control fish (32%). Serum bactericidal activity was significantly higher in probiotic-supplemented fish than in control fish at 14 days post infection. The result study suggests that supplementation of *L. rhamnosus* in feed can enhance tilapia immunity against streptococcosis.

Keywords: *Lactobacillus rhamnosus*, *streptococcus iniae*, tilapia

Lactobacillus rhamnosus GG, a potential human-derived probiotic candidate for tilapia (*Oreochromis niloticus*) culture

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Introduction

The use of lactic acid bacteria from human origin as a potential probiotic supplementation in aquaculture feed is now widely contributed. Various health effects of human probiotics have been reported, such as the prevention of acute diarrhea in children, the prevention of antibiotic-associated diarrhea and the prevention and treatment of allergy, as well as the immune stimulation. Therefore, supplementation of human-derived probiotic in aquaculture feed may not only promote aquatic animal's health, but also benefit for human health. While many *Lactobacillus* strains have been promoted as good probiotics for human or animal use, substantial supporting *in vitro* and *in vivo* data are available for only a few in aquatic animals. The present study was designed to investigate some properties and mechanisms of action of *Lactobacillus rhamnosus* GG, originated from human on growth performance and humoral and cellular immunity in tilapia (*Oreochromis niloticus*).

2 Materials and methods

2.1 Fish and probiotic supplementation

One hundred tilapias, *Oreochromis niloticus*, 30-50 g body weight, were allowed to acclimatize for 7 days and were randomly placed in two 60-L tanks. The probiotic bacterium, *Lactobacillus rhamonsus* GG (ATCC 53103), was cultured in MRS broth at 30 °C for 48 hrs, the bacteria were manually incorporated into commercial dry pellets at the rate of 10^{10} CFU/g in feed for probiotic group. Fish fed only commercial dry pellets served as a control. Fish were

fed approximately 1.5 % of body weight once a day. One month after feeding, fish were sampled for health parameters. The experiment was duplicated at the different time point.

2.2 Growth performance

For growth performance parameters, weight gain (%), specific growth rate and feed conversion ratio (FCR) were calculated by using the following equations (Yanbo and Zirong, 2005):

Weight gain (%) = 100 X (final mean body weight-initial mean body weight) / initial mean body weight

Specific growth rate = [(ln (final body weight) – ln (initial body weight) / days] x 100

Feed conversion ratio= feed intake (g) / Weight gain

3 Immunological aspects

3.1 Preparation of head kidney leukocytes

Head kidneys and blood were collected from 6 fish in each group at 14 days. The head kidneys were rinsed with RPMI1640 and separated through a 100 µm metal mesh into RPMI1640 medium using silicon tip to dislodge the leukocytes. Two ml of percoll solution (1.079 g/l) were added and spun at 350-400 g for 20 minutes at 4°C. The leukocytes were obtained from the interface and washed twice with RPMI1640 (10%FBS). The number of leukocytes was counted using a haemocytometer with Tryphan blue inclusion. The experiment was continued upon the survival of 95 % of leukocytes for chemiluminescence assay. The blood samples were obtained by using syringes through the caudal vessels and centrifuged to make the sera for lysozyme and complement activity assay.

3.2 Phagocytic activity

3.3 Chemiluminescence assay of leukocytes

The 1 x 10^7 cells/ml leukocytes used for this assay were incubated for three minutes at 28°C, the contents were spun down and the pellets were resuspended with 50 µl of RPMI-1640. The CLA (2-methyl-6-phenyl-3, 7-dihydroimidazo [1,2-a] pyrazine-3-one) dependent chemiluminescence was assayed in a lumi-counter (Microtech Nichion NU2500, Japan) by mixing 50 µl leukocytes, 100 µl CLA, 300 µl PBS (-) and 50µl phorbol 12-myristate 13-acetate (1µg/ml). The peak value was recorded and tested for statistical analysis.

3.4 Serum lysozyme activity

The blood samples were obtained by using syringes through the caudal vessels and centrifuged. Lysozyme activity in serum was assayed according to the method of Demers and Bayne (1997) based on the lysis of the lysozyme sensitive gram positive bacterium, *Micrococcus lysodeikticus* (Sigma). The change in turbidity was measured every 30 s for 5 min at 450 nm using a microplate reader.

3.5 Alternative complement activity (ACH50)

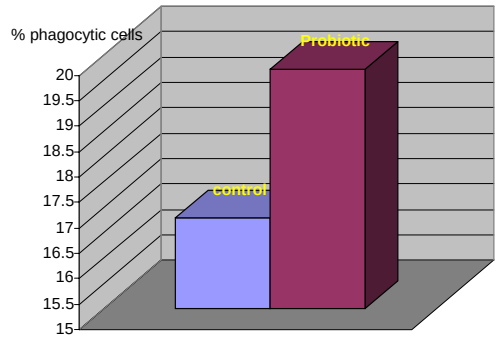
The alternative complement activity was determined following Yano (1992) by using rabbit red blood cells (RaRBC). The optical density of the supernatant was measured at 414 nm using a DU 640 spectrophotometer (Beckman Instruments Inc., California, USA). A lysis curve was obtained by plotting the percentage of haemolysis against the volume of serum added. The volume yielding 50% haemolysis was determined and in turn used for assaying the complement activity of the sample (ACH50 value = units/ml).

Results and Discussions

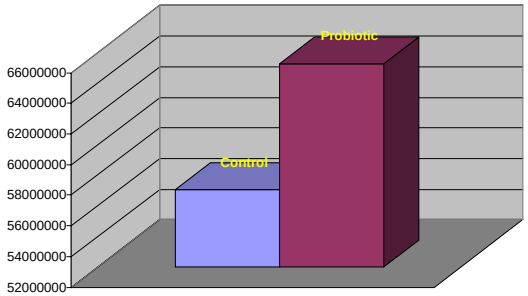
Growth performance

Growth performance	Control	Probiotic
Initial mean body weight	37.02	35.76
Mean body weight (14 d)	44.82	44.12
Mean body weight (30 d)	49.16	49.38
Weight gain (%) 14 days	21.07	23.38
Weight gain (%) 30 days	32.79	38.09
Specific growth rate (14 d)	1.44	1.46
Specific growth rate (30 d)	1.03	1.08
Feed conversion ratio	1.37	1.18

Phagocytic activity

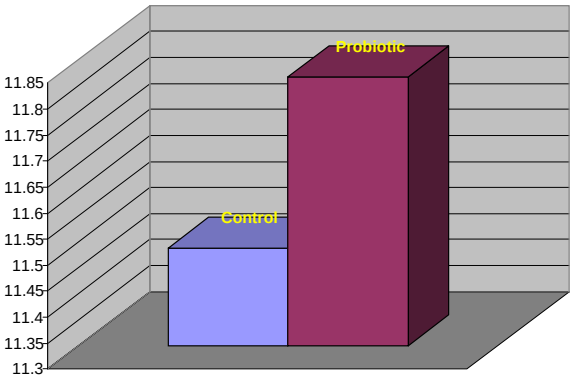


Chemiluminescence assay of leukocytes



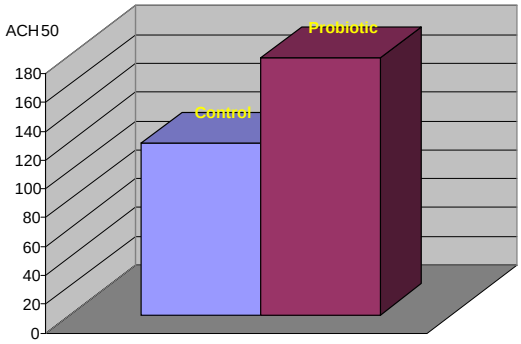
Head kidney leukocytes had significantly greater activity in probiotic fish when compared with control fish

Lysozyme activity



Serum lysozyme activity was rather high in both group. No significantly statistic analysis was observed between the probiotic and control group.

Alternative complement activity



ACH50 activity was significantly higher in the probiotic supplemented groups than in the control group.

Discussion

The study confirmed that dietary supplementation with *L. rhamnosus* GG enhanced the growth performance and immunity of tilapias. Increase phagocytic activity, serum lysozyme, complement activity and killing ability suggest that *L rhamnosus* indeed provides protective effect against harmful pathogens or serious diseases. The evidence of *L rhamnosus* induced innate defense mechanism both humoral and cellular events plays a vital role in preventing diseases in tilapia.

Acknowledgement

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The efficacy of *Lactobacillus rhamnosus* GG against Streptococcosis in tilapias (*Oreochromis niloticus*): Growth performance and protective effects

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อาจารย์ที่ปรึกษาโครงการ

ผศ.น.สพ.ดร.นพดล พิพารัตน์

อาจารย์ที่ปรึกษาโครงการร่วม

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รายงานนี้เป็นส่วนหนึ่งของโครงการเสริมทักษะการวิจัย (3100610)
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ประสิทธิภาพของเชื้อโปรไบโอติก (*Lactobacillus rhamnosus*) ต่อการป้องกันโรค Steptococcosis และการเจริญเติบโตในปลานิล

บทคัดย่อ

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ในปัจจุบันการใช้โปรไบโอติกผสมอาหารได้รับความสนใจมากขึ้นเพื่อใช้ในการควบคุมโรคต่างๆในปลา การศึกษานี้ได้ศึกษาประสิทธิภาพของโปรไบโอติก (*Lactobacillus rhamnosus*) ต่อการเจริญเติบโตของปลานิลและการควบคุมโรคที่เกิดจากเชื้อ *Streptococcus iniae* และเชื้อ *Streptococcus agalactiae* โดยใช้เชื้อปริมาณ $2x10^8$ เซลล์ต่อมิลลิลิตร ฉีดเข้าช่องท้อง ผลการทดลองการให้อาหารเสริมโปรไบโอติกเป็นระยะเวลา 14 และ 30 วัน พบว่ากลุ่มโปรไบโอติกมีการเพิ่มขึ้นของน้ำหนักตัว อัตราการเจริญเติบโต และ อัตราการแลกเนื้อดีกว่ากลุ่มควบคุม นอกจากนี้ในกลุ่มโปรไบโอติกยังมีความยาวของวิลไลมากกว่ากลุ่มควบคุมอีกด้วย ในการทดสอบความสามารถในการยับยั้งจุลชีพในห้องปฏิบัติการด้วยวิธี agar spot พบว่า *L. rhamnosus* สามารถยับยั้งการเจริญเติบโตของเชื้อ *S. iniae* (4 สายพันธุ์) และเชื้อ *S. agalactiae* (2 สายพันธุ์) เมื่อทำการฉีดเชื้อพิษ (เชื้อ *S. iniae* และเชื้อ *S. agalactiae*) เข้าปลานิล พบว่ากลุ่มโปรไบโอติกมีเปอร์เซ็นต์การรอดชีวิตสัมพัทธ์ (RPS) เท่ากับ 62.5 และ 46.43 ตามลำดับ ในทางจุลพยาธิวิทยาพบว่า กลุ่มโปรไบโอติกมีจำนวน melano-macrophage center มากขึ้นบริเวณไตส่วนหน้าในวันที่ 3 และ 7 หลังการฉีดเชื้อ จากผลการทดลองพบว่าการเสริมโปรไบโอติก (*L. rhamnosus*) ในอาหารสามารถเพิ่มการตอบสนองของระบบภูมิคุ้มกันต่อโรคสเตรปโตคอคโคซิสและเพิ่มการเจริญเติบโตได้ในปลานิล

คำสำคัญ: โปรไบโอติก *Lactobacillus rhamnosus* การเจริญเติบโต ความสามารถในการยับยั้งจุลชีพ ระบบภูมิคุ้มกัน

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The efficacy of *Lactobacillus rhamnosus* GG against Streptococcosis in tilapias (Oreochromis niloticus): Growth performance and protective effects

Abstract

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Probiotic supplementation is now being focused as an alternative method to control fish diseases. This study investigated the efficacy of the probiotic in growth performance and the protective effects of *Lactobacillus rhamnosus*-supplemented diet against *Streptococcus iniae* and *Streptococcus agalactiae* after intraperitoneal challenging at a concentration of $2x10^8$ CFU/fish. After feeding for 14 and 30 days, the probiotic group had better weight gain, specific growth rate and feed utilization (FCR). In addition, the villus height in probiotic group was greater than control group. An *in vitro* study on antimicrobial activity using agar spot test and disc diffusion showed that *L. rhamnosus* strongly inhibited the growth of *S. iniae* (four strains) and *S. agalactiae* (two strains). In the *in vivo* study, the relative percent survival (RPS) in the probiotic group challenged with *S. iniae* was 62.5 and RPS in the probiotic group challenged with *S. agalactiae* was 46.43. Histopathology, both of probiotic groups had more evidence in number of melano-macrophage center in head kidney at 3,7 DPI. The results of this study suggested that supplementation of *L. rhamnosus* in feed could enhance immunity against streptococcosis and enhance the growth performance in tilapias.

Key word: Probiotic, *Lactobacillus rhamnosus*, growth performance, antimicrobial activity, immunity

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Introduction

Streptococcus sp. is a gram-positive, encapsulated, facultative anaerobic cocci bacterium in pairs or chains. The bacteria in this genus cause serious diseases in a number of different hosts such as mammals, fish and also humans. In aquatic animals, the disease can occur in both wild and cultured fish. In 1957, the first identification of *Streptococcus* sp. in cultured fish (rainbow trout) in Japan was reported (Hoshina *et al*., 1958). The bacteria had been identified from various parts of the world particularly in intensive culture. In addition, when an outbreak occurred it made a serious economic loss due to high morbidity and mortality (Bromage and Owens, 2002). *S. agalactiae* and *S. iniae* are the two species that are most reported. They tend to cause different stages of disease depend on their host. Both of them cause similar clinical signs - spiral or erratic swimming, ocular abnormalities such as peri-orbital and intraocular haemorrhage, opacity and exophthalmia, reddened or haemorrhage in integumental and muscoskeletal systems, ascites and ulceration(Bromage and Owens, 2002). However, in some cases the fish may not show any clinical signs before death.

Traditional classification of Streptococci is based on serogrouping of carbohydrate antigens of the cell wall and their haemolytic activities. *S. agalactiae* is a group B Streptococcus that can be either haemolytic or non-haemolytic on a blood agar plate. It has been recognized as causing mastitis in bovine, neonatal meningitis, sepsis, and pneumonia in humans. Although *S. agalactiae* is found in both humans and animals, zoonotic transmission seems to be nonexistent or insignificant. The strains that cause disease in humans are usually biochemically, metabolically or serologically different from the strains that cause animal diseases. The first isolation of *S. agalactiae* in fresh water fish was from golden shiners (*Notemigonus crysoleucas*) (Robinson and Meyer, 1966). *S. iniae* is a non-group *Streptococcus* and always hemolytic on a blood agar plate. *S. iniae* was first identified from multiple subcutaneous abscesses in freshwater dolphins. Like *S. agalactiae*, they can cause disease in fish, mammals, and also humans, but in contrast, *S. iniae* is zoonotic. *S. iniae* infections in humans have occurred mainly in people with skin injuries caused by handling live or fresh fish. In humans, it brings about the development of cellulitis, which is occasionally localized in organs or joints (Facklam *et al*., 2005; Lau *et al*., 2003). The conventional methods to prevent and control Streptococcosis are chemotherapy and vaccination. Regarding vaccination, there was a report of vaccination of *S. iniae* in rainbow trout farms from 1995 to 1997 with good results in decreasing the mortality from 50% to 5%, but shortly after vaccination, massive new outbreaks occurred (Bachrach *et al.*, 2001). Until present, there has not been any commercial vaccine that can give satisfactory results. Although using antibiotics

is an effective way for treating infected fish, drug resistance usually happens amongst dense populations (Agnew and Barnes, 2007). Furthermore, consumers are much more concerned about food safety than in the past.

"A live microbial feed supplement which beneficially affects the host animals by improving its intestinal microbial balance" (Fuller, 1989) is a common term of probiotic in the past which sometimes added "mono- or mixed culture of live microorganisms"(Havenaar and Huis in't Veld, 1992) and "microbial cell preparations or components of microbial cells" (Salminen *et al*., 1999). For aquaculture, Gatesoupe (1999) redefined the word probiotic as "Microbial cells that are administered in such a way as to enter the gastrointestinal tract and to be kept alive, with the aim of improving" and Verschuere *et al*., 2000, gave additional definitions including "The ability of a probiotic to modify the "Host-associated or ambient microbial community" and "To improve the quality of its surroundings, both of which can be considered as biocontrol." In aquaculture, there are many studies mentioning the efficacy of probiotics as a growth promoter, for example, some treatments with lactic acid bacteria increased the production of rotifers and the growth of turbots and Japanese flounders (Gatesoupe, 1989, 1991); *Bacillus subtilis* and *Lactobacillus acidophilus* as a dietary supplement gave a greater growth performance in tilapias (Aly *et al*., 2008). The principle actions of probiotics that brings out better growth performance are reducing stress, protecting a host from intestinal disorder by preventing adhesion from pathogen, inhibiting pathogenic microorganisms, enhancing the host immune response(Fuller, 1989; Gatesoupe, 1999; Verschuere *et al*., 2000) and improving nutrition (Balcazar *et al*., 2006; Fuller, 1989; Planas *et al*., 2004; Suzer *et al*., 2008).

The key to success of preventing and controlling infectious diseases depends on several factors; the host immunity, the pathogen and also the environment. In the aquatic system, which the hosts and the pathogens share the same ecosystem, the hosts are fully exposed to the pathogens (Verschuere *et al*., 2000). The use of probiotic bacteria has become an interesting alternative way because of their ability of inhibiting growth of other microorganisms and modulating the host immunity (Fuller, 1989; Gatesoupe, 1999; Verschuere *et al*., 2000). The direct effect such as inhibiting growth of other organisms might be the main action that could occur in cultured system (Kesarcodi-Watson *et al*., 2008). 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). Since Lactic acid bacteria(LAB) probiotics are considered safe for food fish and have ability to fight against harmful pathogens directly and indirectly, they are now

being used as an alternative method to control diseases. *Lactobacillus rhamnosus*(Lactic acid bacteria) is a human-derived probiotic that has been used in humans to control gastrointestinal disease and some bacterial-infection diseases.

This study was conducted to study the efficacy of a human-derived probiotic, *L. rhamnosus*, in growth performance and the ability to fight against aquatic pathogenic bacteria, *S. iniae* and *S. agalatiae* in tilapia.

Materials and methods

Probiotic and pathogenic bacteria

L. rhamnosus(ATCC 53103) was cultured at 37º C on De Man, Rogosa and Sharpe (MRS)(Difco™, France)(a suitable media for lactic acid bacteria) De Man *et al.,*1960) agar with 0.3% $CaCO_3$. Four strains of *S. iniae* and two strains of *S. agalactiae* from laboratory collection confirmed by PCR were cultured at 30 º C on Tryptic soy agar(TSA) a suitable media for pathogen bacteria.

***In vitro* antimicrobial activity**

An agar spot test was used to screen for antibacterial properties of *L. rhamnosus* against pathogenic bacteria. In a separate experiment, *L. rhamnosus* was spotted on the surface of Modified-MRS agar (2g dextrose for decrease producing lactic acid) and TSA.

An agar well diffusion assay and an agar spot test with killed probiotic bacteria were used to find out what element/s was/were the cause/causes of the inhibition.

Agar spot test

L. rhamnosus from an overnight culture(24h.) in MRS broth was spotted on the surface of M-MRS agar and TSA incubated at 37°C for 24 hours to allow the development of the colonies. After 24 h., 50 µl of each strain of *S. iniae* and *S. agalactiae* (5×10^6cfu/ml) were 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 (spotted with grown *L. rhamnosus*). The plates were incubated at 30°C for 24 h. and checked for inhibition zone. (n=8, Data were expressed as a mean ± S.D)

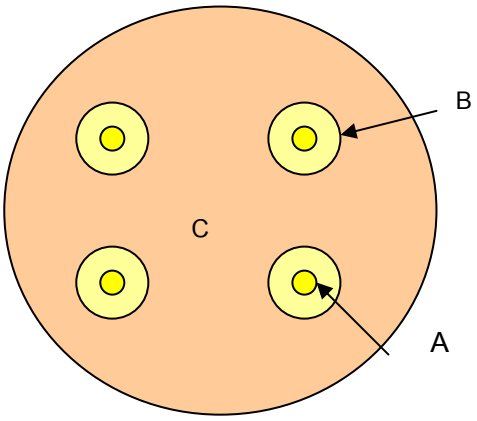

Fig 1 Agar spot test: A=*L. rhamnosus*, B=inhibition zone, C= pathogenic bacterium

Agar spot test with killed probiotic bacteria

L. rhamnosus from an overnight culture in MRS broth was centrifuged at 5,000g 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 cells were spotted on the surface of M-MRS agar and overlaid with each strain of *S. iniae* and *S. agalactiae* in the same method as above. The plates were incubated at 30°C for 24 h. and checked for inhibition zone. (n=8, Data were expressed as a mean ± S.D)

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

Five sterile blank paper discs were placed on the Muller Hilton agar which was inoculated with strains of *S. iniae* and *S. agalactiae.* Then, 100 µl of the filtered supernatant of *L. rhamnosus* were applied on the paper discs. Plates were incubated at 30°C for 24 h. and observed for inhibition zone. (n=8, Data were expressed as a mean ± S.D)

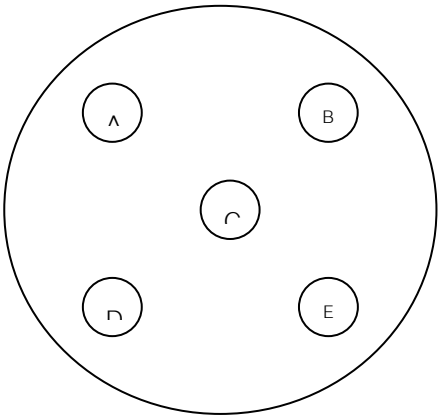

Fig 2 Disc diffusion assay: A=supernatant from M-MRS broth, B=M-MRS broth, C= supernatant from M-MRS broth adjust pH to 6.5, D= MRS broth, E= supernatant from MRS broth

Fish, probiotic supplementation and *in vivo* experimental designs

Two hundred tilapias, *Oreochromis niloticus*, with 20-30 g body weight, were acclimatized for 7 days and were randomly placed in eight 60-L tanks (25 fish per tank) for the control groups (4 groups) and the probiotic groups (4 groups). The tanks were filled with recycled water that was kept at 25˚-28˚ C, 5.8–6.8 ppm dissolved oxygen (DO) and 6.5–7.0 in pH throughout the experiment.

The probiotic bacterium, *L. rhamonsus*, was cultured in MRS broth at 37°C for 48 h, centrifuged and washed with sterile PBS 3 times. Bacterial pellets were measured in PBS and their densities were determined. Under sterile conditions, the bacteria were mixed into commercial dry pellets at the rate of 10^{10} CFU/g (Pirarat *et al*., 2006). The fish in the control group were fed with the commercial dry pellets. They were fed approximately 1.5% of body weight once a day. At day 0, 14, 30 of feeding, the fish were weighed for the growth performance.

After feeding the fish with the probiotic for a month *S. iniae* strain II and one *S. agalactiae* strain I were selected to challenge the fish by injecting 0.2 mL ($2x10^8$ CFU/mL) of the bacterial solution intraperitoneally. Mortality and clinical signs were recorded for 14 days. Re-isolation and immunohistochemistry were used to confirm cause of the dead fish. (Data not shown) This experiment was conducted three times.

For challenge test, relative percent survival (RPS) was calculated follow the equation (Yong *et al*., 2005):

RPS= (1- (Probiotic mortality/control mortality)) x 100

Growth parameters

For growth performance parameters, weight gain (%), specific growth rate and feed conversion ratio (FCR) were calculated by using the following equations (Yanbo and Zirong, 2005):

Weight gain (%) = 100 X (final mean body weight-initial mean body weight)/initial mean Bodyweight

Specific growth rate = [(ln (final body weight) – ln(initial body weight)/days] x 100

Feed conversion ratio= feed intake (g) / Weight gain

Measurement of villus height

After 30 days of feeding, three parts of the intestines, the foregut (after the pyloric part of the stomach to before the spiral part of the intestines), midgut (the spiral part of the intestines) and hindgut (after spiral part of the intestines to 2 cm. before anus), from these fish in the probiotic and control groups were collected and fixed in 10% buffered formalin. Fixed tissues were processed according to standard histological techniques and tissue sections were stained with haematoxylin and eosin (H & E). For villus height measurement, 10 highest villi were selected per section. The villus length was measured from the villus tip to the bottom. An average of these 10 villi per section was expressed as the mean villus height for each section.

Histopathology

For experimental challenge, six tissues (brain, spleen, head kidney, trunk kidney, liver and intestines) of three fish in each group were collected at 0, 3, 7 and 14 days post infection (DPI) and fixed in 10% buffered formalin for histopathology. The unexpected moribund fish in the control group were also sampled. Fixed tissues were processed according to standard histological techniques and tissue sections were stained with haematoxylin and eosin (H & E). The tissue sections were given an arbitrary score from 0 to 3 based on the frequency and severity of the lesions: 0, for no histopathological change; 1, mild; 2, moderate and 3 for severe histopathological change.

Statistical analysis

Data were expressed as a mean ± S.D. and were evaluated using one-way ANOVA followed by the Bonferroni-type multiple t-test. For each histopathology score, the data were subjected to analysis of variance by using a nonparametric Mann–Whitney test. All tests used a significant difference level of $P < 0.05$.

Results

Antimicrobial activity

The agar spot test in both M-MRS and TSA agar showed that all strains of *S. iniae* and *S. agalactiae* had inhibition zones, especially on M-MRS agar (Table1). The widest inhibition zone on M-MRS agar was found in *S. iniae* IV and the widest inhibition zone on TSA was found in *S. iniae* II. There was no inhibition zone in any strains of *S. iniae* and *S. agalactiae* undergoing agar spot test with killed probiotic bacteria. (Data not shown)

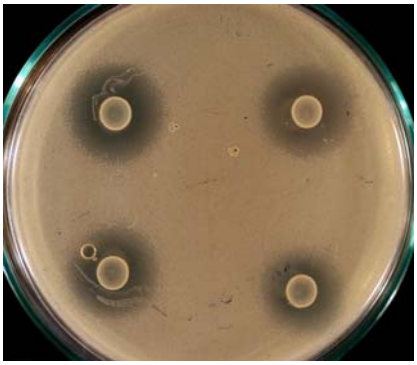

Fig 3 Agar spot test on M-MRS agar with *S. iniae* IV

	Agar spot test				**Disc diffusion**	
	M-MRS		TSA			
	inhibition zone	SD	inhibition zone	SD	inhibition zone	SD
S. iniae I	0.58	0.46	0.25	0.06	0.25	0.1
S. iniae II	0.52	0.46	0.73	0.21	0.8	0.2
S. iniae III	0.52	0.07	0.25	0.14	0.25	0.13
S. iniae IV	1.2	0.96	0.23	0.05	0.23	0.05
S. agalactiae I	0.9	0.23	0.15	0.05	0.15	0.06
S. agalactiae II	0.2	0.07	0.28	0.09	0.275	0.1

Table 1 Inhibition zone of agar spot test and disc diffusion assay (n = 8)

Disc diffusion assay

Cell-free supernatant from 48 h culture of *L.rhamnosus* was able to inhibit the growth of all 4 strains of *S. iniae* and 2 strains of *S. agalactiae.* Strain II of *S. iniae* was the most susceptible. (Table 1)

Fig. 4 Disc diffusion assay A=supernatant from M-MRS broth, B=supernatant from M-MRS broth adjust ph to 6.5,

C=M-MRS broth, D=MRS broth, E=supernatant from MRS broth

Growth performance

Data on the growth performances of the tilapias, including weight gain (%), specific growth rate and feed conversion ratio (FCR) of the tilapias are shown in Fig. 5. The probiotic group showed higher weight gain (%), specific growth rate and feed conversion ratio than the control in the first and second phases of feeding.

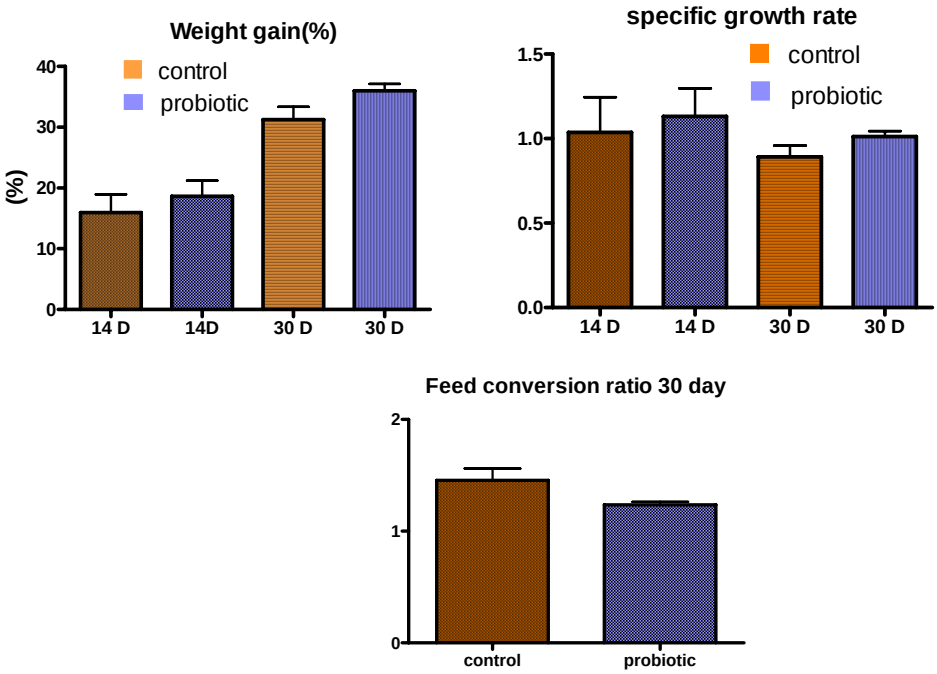

Fig. 5 Growth performance after probiotic feeding at 14 and 30 day(n=3)

14D = after feeding 14 day, 30D = after feeding 30 day

Measurement of villus height

The intestine of both control and probiotic groups were characterized by mucosal folds which appears swollen and bulging towards the lumen that contains numerous mucous cells. The villus height, at the foregut was the highest part followed by the mid-gut and the hindgut. In the foregut, mid-gut and hindgut of the intestines, the villi height of the probiotic group were significantly greater than the control group ($P<0.05$).

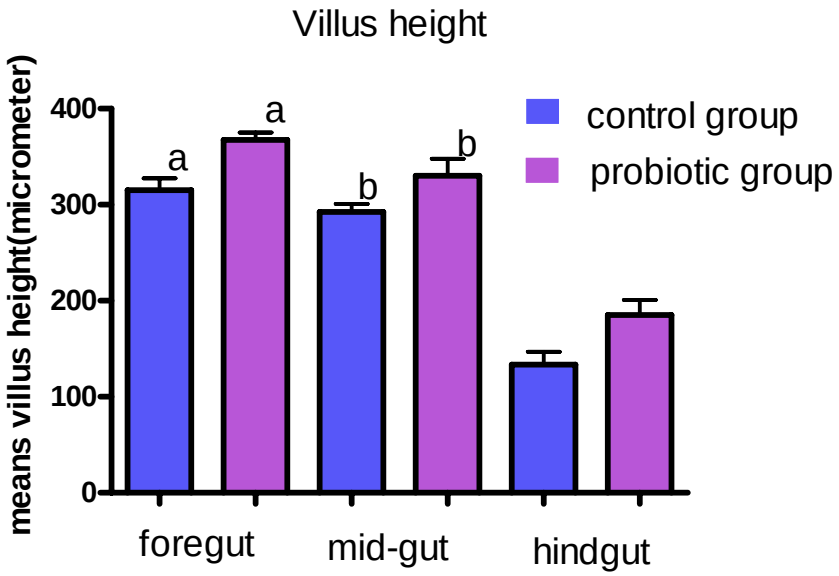

Fig. 6 Villus height between probiotic and control group. Means having the same letter are significantly different at

$P < 0.05$

***S. iniae* and *S. agalactiae* challenge test**

The first mortality of fish in the *S. iniae* challenge trial began at 2 DPI in the control group and on 4 DPI in the probiotic group. Mortality still occurred until 12 DPI in the control and probiotic groups. The highest mortality was recorded at 6 DPI in the control group and at12 DPI in the probiotic group. The relative percent survival (RPS) in the probiotic group was 62.5. The cumulative mortality was significantly lower in the probiotic group (12%) than in the control group (32%). While challenging with *S. agalactiae*, the first mortality began at 1 DPI in the control and probiotic groups. Mortality still occurred until 12 DPI in the control group and until 8 DPI in the probiotic group. The highest mortality was recorded on 3 DPI in the control group and at 2 DPI in the probiotic group. The relative percent survival (RPS) in the probiotic group was 46.43. The cumulative mortality was significantly lower in the probiotic group (16%) than in the control group (34.7%). (Data not shown)

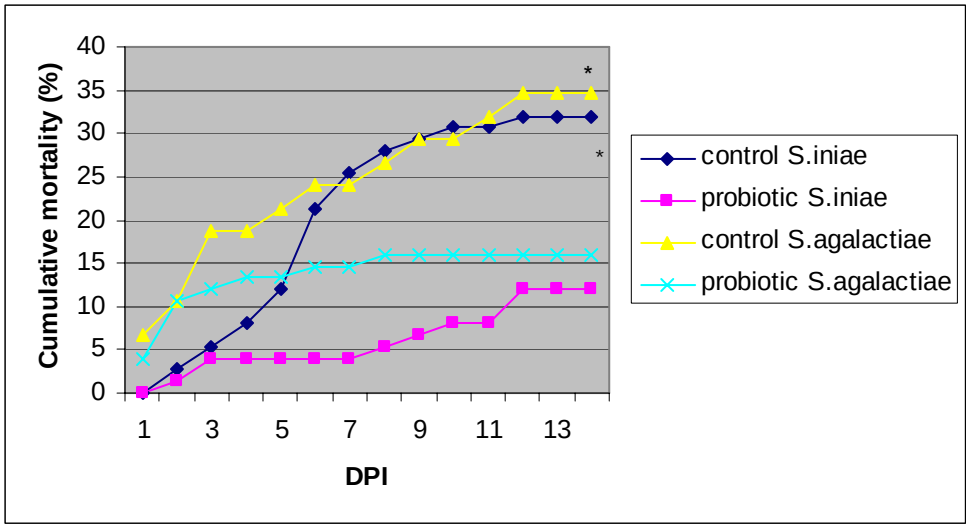

Fig. 7 Average cumulative mortality. * Significant statistical difference among the groups ($P < 0.05$).

Histopathology

The *S. iniae* and *S. agalactiae* challenges caused many kinds of alterations in various organs in both the control and the probiotic groups (Table 1). In the head kidneys and spleens, the increase of melano-macrophage center was very noticeable in both the control and probiotic groups. In the head kidney, at the beginning (3DPI) of the challenge tests, there was found an increase of the melano-macrophage center, which was still higher on 7 DPI and decreased in later stages of infection (14DPI) except in the control group challenged with *S. agalactiae*. In the moribund fish of both challenges, there was also an increase, but significantly in a lower degree when compared with the other groups. In the same way, the spleens also showed an increase of melano-macrophage center after being challenged. There were remarkable lesions of haemolysis of red blood cells in the head kidneys in the probiotic and control groups, but both of the probiotic groups incline to decrease this lesion. The moribund fish of both challenges tend to have more severe lesions than the other groups particularly when challenged with *S. agalactiae*.

In the trunk kidneys, histopathological changes were found more in early stages of infection (before 7 DPI). The renal tubular degeneration characterized by hyaline deposition in tubular cells with the nucleus displaced to the side occasionally with renal tubular necrosis and calcification of renal tubules was discovered in all groups. In the livers, fatty degeneration was found in a low extent and glycogen degeneration was found in a high extent in all four groups. The brains from the probiotic groups of both challenges showed a lower degree of meningitis than from the control groups, while the degree of meningitis in the moribund fish was more severe.

Table 3 Histopathology scores of tilapias infected with *S. iniae* and *S. agalactiae*

	Control SI	Probiotic SI	Control SA	Probiotic SA	M.SI	M.SA
	0 3 7 14	0 3 7 14	0 3 7 14	0 3 7 14		
Head kidney						
MMC	0.33 0.76 2	0.33 1.67 2	0.33 1.33 1.67	0.33 1 2.33	1	1
Heamolysis	0 1.33 2 1.33	0.33 0 0.67 1.67 2	0 1 1 2	2 0 0.67 1.33 1.67	2	2.33
Spleen						
MMC	1 1 1.33 2.67	1 1.33 2.87 1.33	1 1.33 2.67 1.33	1 1.67 2.33 3	2	2.33
Trunk kidney						
T. degeneration	0 1.33 2 1.5	0 1.17 1.83 0.83	0 1.83 2 1	0 0.83 3 2.33	2	2.76
T. regeneration	0 0 1.33 1	0 0 0.33 1	0 0 0.67 0.67	0 0 0 1.33	0	0
Calcification	0 0 1 1.17	0 0.17 1 1.33	0 1.33 0.5 0	0 0.17 0.5 0	0	0
Brain						
Meningitis	0 1 1 1	0 1 0.67 0.67	0 0.67 1 1.33	0 0.33 0.33 0.33	2	2
Liver						
Fatty degeneration	0.33 0.67 0 0	0 1 1 1.67	0.33 0 0 0	0 1 0.67 1	2	1.67
Glycogen degeneration	0 2 1.67 2	0 1.67 2 1.67	0 1.67 1.67 2.33	0 3 2 2	1	2.33

SI=*S. iniae*, Sa=*S. agalactiae*, M.= moribund fish, T.= tubular

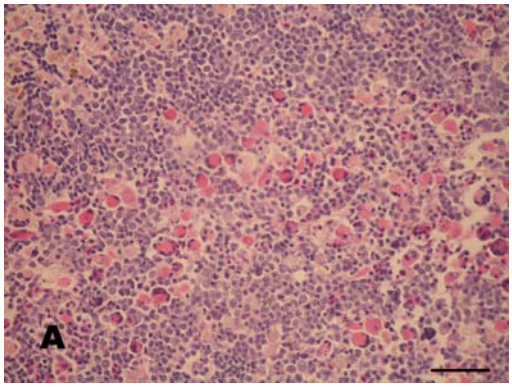

Fig 4 Heamolysis in head kidney from moribund fish challenged with *S. agarlactiae*.
Bar=125μ

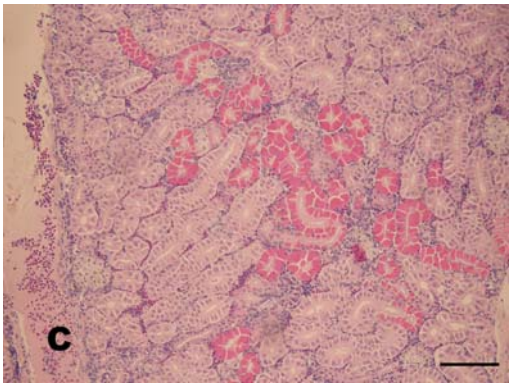

Fig 6 Tubular degeneration from control group challenged with *S. agalactiae*(7 DPI),
Bar=125μ

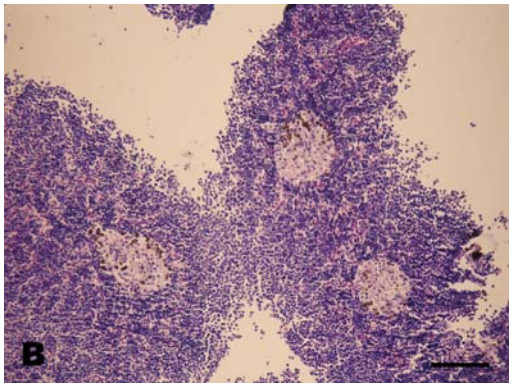

Fig 5 Granuloma-like lesion in probiotic group challenge with *S. agarlactiae*,
Bar= 12.5μ

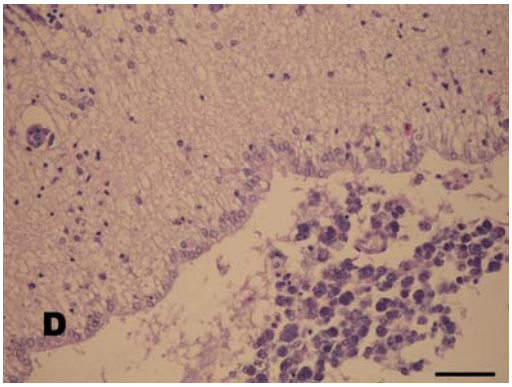

Fig 7 Menigitis in moribund fish challenged with *S. agalactiae*
Bar= 250μ

Discussion

The probiotic-supplemented diet groups resulted in better growth performance and lower FCR than the control groups, which showed that the addition of probiotic can improve the growth performance and feed utilization. Several experiments studied using *Lactobacillus* sp. as the dietary supplement to increase growth performance in various of fish (Suzer *et al*., 2008; Aly *et al.,* 2008). The improvement in fish growth and feed utilization in the probiotic-supplemented group could be induced by the effects of probiotic action, including the maintenance of normal intestinal microflora, improving nutrition by detoxifying the notorious compounds in feeds together with denaturing the potentially indigestible components in the diet by hydrolytic enzymes, including amylases and proteases and producing vitamins such as biotin and vitamin B12 (Balcazar *et al*., 2006; Fuller, 1989; Planas *et al*., 2004; Suzer *et al*., 2008).In addition, the morphology of the villi is the important thing that can point out the growth performance. The increase in the length of the villi implies an increase of surface area for greater absorption of available nutrients (Caspary, 1992). In this present study, the probiotic group had longer in villus height in all parts of the intestines, significantly in the foregut and midgut. This corresponds with the longer villi in chickens and turkeys treated with *L. reuteri* (Dunham *et al*., 1993). Besides, probiotics have proven to induce gut epithelium cell proliferation in rats (Ichikawa *et al*., 1999). The possible mechanism could be that after the probiotic bacteria are transited to the stomach, they germinate in the intestines and use a large amount of sugar for their growth, producing substance that contribute to make short chain fatty acids, which might play an important role in increasing the villus height (Pelicano *et al*., 2005). Short chain fatty acid particularly butyric acid is the main energy source for colonic epithelium cells and able to stimulate the release of gastrointestinal peptide or growth factors which may affect cell proliferation. (Blottiere *et al*., 2003)

The present study on the agar spot test on showed the efficacy of the antimicrobial activity of *L. rhamnosus* over all strains of *S. iniae* and *S. agalatiae*. The agar spot test using killed probiotic bacteria, which showed no inhibition zones, clearly showed that only the metabolite products, not the cells of probiotic bacteria, are involved in the growth inhibition of *S. iniae* and *S. agalatiae*. Lactic acid bacteria are known for their ability to produce inhibitory substance such as hydrogen peroxide, organic acid and bacteriocin-like products, which are antimicrobials (De Vuyst and Leroy, 2007). Moreover from result of disc diffusion assay which showed the inhibition zone only when applied the supernatant from MRS broth and it may suggest that

antimicrobial substances could be from lactic acid bacteria because the main metabolite product from MRS while using M-MRS broth decrease producing lactic acid from *L.rhamnosus*. In addition, the result from the agar spot test on TSA, which is not suitable media for lactic acid, might suggest that even in an unfavorable environment *L. rhamnosus* still can produce antimicrobial substrates.

The efficacy of *L. rhamnosus* was determined from the *in vivo* model. The relative percent survival rate (RPS) in the probiotic group challenged with *S. iniae* is 62.5, which indicates that the probiotics could protect the fish from infection (according to European Pharmacopeia). Although, it gave an unsatisfactory effect in the *S. agalactiae* challenged groups (RPS is 46.43), but still tended to decrease the mortality in the probiotic group. From the previous studies, *L. rhamnosus* has been proven for its potential to control infection from *Aeromonas salmonicida*, *Vibrio anguillarum* and *Flavobacterium psychrophilum* in rainbow trouts and turbots (Nikoskelainen *et al*., 2001). However, it is not clear how probiotics are involved this response. Panigrahi *et al,* 2004 studied on *L. rhamnosus* JMC1136 and found that it can stimulate non specific immune like phagocytosis, lysozyme and complement activities in rainbow trout. Another study showed similar results of *L. rhamnosus* enhancing the alternative complement, enabling phagocytic cell aggregation and increasing phagocytic activity (Pirarat *et al*., 2006). On histopathology in this study both challenges with *S. iniae* and *S. agalectiae* gave the same evidence of melano-macrophage centers in the head kidneys and spleens. In early stages of infection (before 7DPI), the probiotic-dietary supplement group had a higher degree of melano-macrophage centers than the control group. The melano-macrophage center in fish enable the functions of macrophages in domestic animals, phagocytes foreign materials, including infectious agents (Agius and Roberts, 2003). From histopathology, increasing of melano-macrophage center in head kidney both of probiotic groups could assume that using probiotic as a dietary supplement can enhance the immune respones

The data from our experiment clearly showed that the live *L.rhamnosus* a human -derived probiotic can use as probiotic in tilapia and has ability to improve the growth performance and protect the fish from Streptococcosis.

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